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(71) Applicant: UNIVERSITY OF FLORIDA [US/US]; 223 Grinter Hall, Gainesville, FL 32611 (US).

(72) Inventors: AGARWAL, Anupam; 4015 N.W. 59th Avenue, Gainesville, FL 32653 (US). NICK, Harry, S.; 6324 N.W. 97th Court, Gainesville, FL 32653 (US). VISNER, Gary, A.; R.R. 2, Box 430-B, Alachua, FL 32615 (US).

(74) Agents: PACE, Doran, R. et al.; Saliwanchik, Lloyd & Saliwanchik, P.A., Suite A-1, 2421 N.W. 41st Street, Gainesville, FL 32606-6669 (US).

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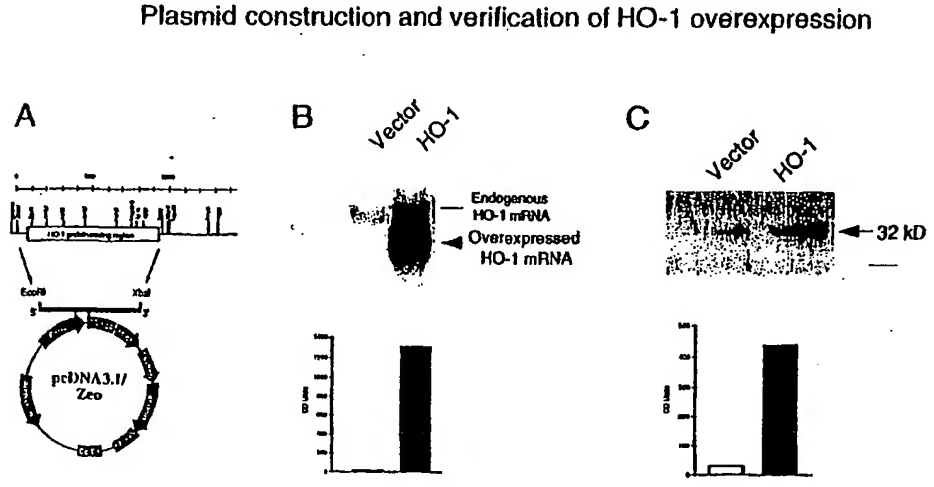

(57) Abstract

The subject invention concerns a novel process for treating a human or animal to prevent cellular or tissue injury when said human or animal is at risk for heme protein-related damage or injury, such as acute renal failure that is associated with cisplatin treatment of cancer patients. The process employs a novel vector which, via *in vivo* gene transfer, results in the overexpression of the HO-1 gene in the kidney. The subject invention also concerns polynucleotide vectors and plasmids comprising the polynucleotide sequences that encode an HO-1 enzyme. These vectors can be used for transforming kidney cells to express the HO-1 gene.

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DESCRIPTION

MATERIALS AND METHODS FOR PREVENTING CELLULAR INJURY IN HUMANS AND ANIMALS

This invention was made with government support under United States National Institutes of Health Grant No. NIDDK K08 DK02446-01. The government has certain rights in this invention.

Cross-Reference to Related Applications

This application claims the benefit of U.S. Provisional Application Serial No. 60/063,863, filed October 31, 1997 and U.S. Provisional Application Serial No. 60/105,400, filed October 23, 1998.

Background of the Invention

Damage to organs, tissues and cells can arise when heme proteins, such as hemoglobin and myoglobin, escape from the intracellular space. Heme oxygenase - 1(HO-1) is a 32 kDa microsomal enzyme that is important in the degradation of heme. HO-1 catalyzes the conversion of heme to biliverdin, releasing iron and carbon monoxide. Biliverdin is subsequently converted to bilirubin which is then excreted from the body. HO-1 expression is inducible not only by the heme substrate, but also by a variety of agents that are associated with, or that cause oxidative stress. A human HO-1 cDNA nucleotide sequence, and the amino acid sequence encoded thereby, has been determined (Yoshida, T., P. Biro, T. Cohen, R.M. Muller, and S. Shibahara (1988) "Human heme oxygenase cDNA and induction of its mRNA by hemin" *Eur. J. Biochem.* 171(3):457-461; Shibahara, S., M. Sato, R.M, Muller, T. Yoshida. (1989) "Structural organization of the human heme oxygenase gene and the function of its promoter" *Eur. J. Biochem.* 179: 557-563; Takeda, K., S. Ishizawa, M. Sato, T. Yoshida, S. Shibahara. (1994) "Identification of a cis-acting element that is responsible for cadmium-mediated induction of the human heme oxygenase gene" *J.Biol. Chem.* 269: 22858-22867).

The literature suggests that the induction of heme oxygenase-1 is an adaptive and

protective response to a wide variety of stimuli in cells and tissues. Most of these studies utilized chemical inducers and inhibitors of HO-1 to establish a functional role for HO-1 in cytoprotection. Previous studies have demonstrated that chemical inducers of the HO-1 gene demonstrate significant protective responses, while inhibition of the HO-1 gene with chemical inhibitors worsen cell injury both *in vivo* and *in vitro* (Agarwal, Anupam, József Balla, Jawed Alam, Anthony J. Croatt, Karl A. Nath (1995) "Induction of heme oxygenase in toxic renal injury: A protective role in cisplatin nephrotoxicity in the rat" *Kidney International* **48**:1298-1307; Shiraishi, Fumie, Harry S. Nick, C. Craig Tisher, Agarwal, Anupam, (1997) Abstract - 30th Annual Meeting (November 2-5, 1997) "Prior Induction Of Heme Oxygenase-1 Attenuates Cisplatin-Mediated Toxicity In Human Proximal Tubule Cells (HPTC)"; and Nath, Karl A., Gyorgy Balla, Gregory M. Vercellotti, József Balla, Harry S. Jacob, Michael D. Levitt and Mark E. Rosenberg (1992) "Induction Of Heme Oxygenase is a Rapid, Protective Response in Rhabdomyolysis in the Rat" *J. Clin. Invest.* **90**:267-270). However, these chemicals may have nonspecific effects other than the induction of HO-1.

Studies have shown overexpression of HO-1 to be protective in toxicity against heme and hemoglobin (Abraham, N.G., Y. Lavrovsky, M.L. Schwartzman, R.A. Stoltz, R.D. Levere, M.E. Gerritsen, S. Shibahara, A. Kappas (1995) "Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: Protective effect against heme and hemoglobin toxicity" *Proc. Natl. Acad. Sci. USA* **92**:6798-6802), and hyperoxia (Lee, Patty J., Jawed Alam, Gordon W. Wiegand, Augustine M.K. Choi (1996) "Overexpression of heme oxygenase-1 in human pulmonary epithelial cells results in cell growth arrest and increased resistance to hyperoxia" *Proc. Natl. Acad. Sci. USA* **93**:10393-10398). More recently, Abraham *et al.* (Abraham, Nader G., Jean-Louis da Silva, Yan Lavrovsky, Robert A Stoltz, Attallah Kappas, Michael W. Dunn and Michael Laniado Schwartzman (1995) "Adenovirus-Mediated Heme Oxygenase-1 Gene Transfer Into Rabbit Ocular Tissues" *Investigative Ophthalmology & Visual Science* **36**(11):2202-2210) have demonstrated a tissue-selective functional transfer of the human HO-1 gene into rabbit ocular tissues *in vivo*. Microinjection of an adenovirus-HHO construct mixed with lipofectamine into the eye resulted in HO-1 mRNA expression in different regions of the eye, without any expression in extraocular tissues such as the brain, liver or kidney. However, there are no studies in the literature that establish targeted gene

delivery of HO-1 to the kidney. Expression of heme oxygenase-1 has also recently been implicated in mouse cardiac xenograft survival in rats (Soares, M.P., Lin, Y., Anrather, J., Csizmdia, E., Takigami, K., Sato, K., Grey, S.T., Colvin, R.B., Choi, A.M., Poss, K.D., and Bach, F.H. (1998) "Expression of heme oxygenase-1 can determine cardiac xenograft survival" *Nature Medicine* 4:1073-1077).

Patients receiving medications for infection (*e.g.*, gentamicin), cancer (*e.g.*, cisplatin), immunosuppression (*e.g.*, cyclosporin), or radiocontrast agents have a high risk for sustaining damage to cells and tissues, such as, for example, damage to kidneys which can result in acute renal failure. Treatment for acute renal failure is expensive, necessitating dialysis and prolonged hospital stay. There is an increase in morbidity and mortality as well. Accordingly, there remains a need in the art for therapies that can prevent or ameliorate cell/tissue damage and/or failure associated with release of heme proteins.

Brief Summary of the Invention

The subject invention concerns methods for providing a person or animal with cytoprotection from heme protein-related cell damage and oxidative stress. Specifically, the subject method pertains to gene therapy using polynucleotide expression vectors or plasmids that include a nucleotide sequence encoding HO-1. Cells or tissue transformed with the subject vectors or plasmids overexpress HO-1 enzyme which catalyzes degradation of heme proteins.

The subject invention also concerns novel polynucleotide expression vectors and plasmids comprising a polynucleotide sequence encoding HO-1. In one embodiment, an expression vector comprising a cytomegalovirus promoter/enhancer and the protein coding region of the HO-1 gene (pcDNA3.1/HO-1) is exemplified. When transfected into target cells, vectors of the subject invention provide for overexpression of HO-1 in those cells.

The targeted expression system of the invention can be used as a therapeutic, and more importantly, as a preventative modality in treatments and procedures that present high risk for heme protein-related cytototoxicity or oxidative stress in a patient.

Brief Description of the Drawings

Figure 1A shows construction of a plasmid designated as pcDNA3.1/Zeo. **Figure 1B** shows a Northern blot of RNA from human renal tubule cells transfected with the pcDNA3.1/Zeo plasmid encoding the HO-1 enzyme. **Figure 1C** shows a Western blot of proteins from human renal tubule cells transfected with the pcDNA3.1/Zeo plasmid encoding the HO-1 enzyme. The Western blot confirms the presence of a 32 kD protein.

Figure 2 shows cytoprotective effects of transient overexpression of the HO-1 gene in human proximal tubule cells exposed to cisplatin.

Figure 3 shows micrographs of human renal tubule epithelial cells that are either (panel A) untreated control, (panel B) treated with 100 μM cisplatin, or (panel C) pretreated with hemin and then challenged with 100μM cisplatin.

Figure 4A shows the amino acid sequence (in standard single letter code) of the human HO-1 enzyme. **Figure 4B** shows a cDNA sequence encoding the HO-1 enzyme.

Figure 5 shows overexpression of HO-1 decreases cisplatin mediated toxicity as measured by LDH assay.

Figure 6 shows cytoprotective effect of hemin pretreatment in proximal tubule cells exposed to cisplatin.

Figure 7 shows the effect of hemin pretreatment on cisplatin-induced apoptosis in human proximal tubule cells.

Figure 8 shows the effects of overexpression of HO-1 in HEK293 cells exposed to cisplatin as measured by mitochondrial viability by the MTT assay.

Detailed Disclosure of the Invention

The subject invention concerns methods and materials for protecting cells and tissues at risk for heme protein-related damage and/or exposure to oxidative stress. In one embodiment, a method of the subject invention comprises gene therapy of targeted cells or tissues to introduce polynucleotide sequences which when expressed result in overexpression of an enzyme having HO-1 activity. Cells and tissues contemplated for treatment by the subject methods include, for example, kidney, spinal cord, brain, liver, lung, intestine and skin.

Specifically contemplated within the scope of the present invention are methods

for treating cancer patients, transplant patients and others that are to receive treatments with injurious agents, such as cisplatinin and cyclosporine, that are associated with heme protein or oxidative-related damage to normal cells and tissues in the patient. In one embodiment, the subject method comprises utilizing gene therapy techniques to introduce polynucleotide sequences into targeted cells or tissues which when expressed in those cells or tissues result in overexpression of an HO-1 enzyme, or a biologically active fragment or variant thereof.

In one embodiment, the present invention concerns a method for providing overexpression of HO-1 protein to provide cytoprotection in human renal proximal tubule kidney cells. Cells in the proximal tubule of the nephron are most susceptible to a wide variety of injurious agents, *e.g.*, drugs (gentamicin, cisplatin, cyclosporine), contrast agents used in radiologic procedures, environmental toxins (cadmium) and ischemia-reperfusion renal injury. One embodiment of the subject method provides a polynucleotide expression vector comprising at least that region of an HO-1 gene that encodes an HO-1 enzyme, or a biologically active fragment or variant thereof, to transform kidney cells to express HO-1 in a patient in need of such treatment. Once the cells have been transformed with a polynucleotide vector of the present invention, the HO-1 gene is overexpressed in those cells. In a preferred embodiment, the expression vector is integrated into the genome of the target cell to provide stable overexpression of an HO-1 enzyme in those cells.

Polynucleotide expression vectors of the present invention can be introduced into cells or tissues by *in vivo* or *ex vivo* means. Preferably, the polynucleotide vectors are introduced *in vivo* by a variety of viral and non-viral means. Suitable nonviral means include, for example, polynucleotide complexed with cationic lipids, and encapsulation of the polynuceotides in liposome vesicles. Suitable viral vectors can be prepared from retroviruses, such as murine leukemia viruses and HIV-derived vectors , adeno-associated viruses, and adenovirus. Other suitable viral vectors are known in the art and are contemplated in the present invention.

Polynucleotide vectors of the present invention for HO-1 gene therapy can be administered to a person in need of such treatment according to standard procedures and methods known in the art. Preferably, the polynucleotide vectors are administered in a biologically compatible solution by direct injection or contact with the target cells and/or

tissue of the patient. For example, the subject vectors can be administered to protect kidney cells from damage from cisplatin and other agents, by injecting *in vivo* a polynucleotide expression vector directly in kidney tissue or by contacting kidney tissue *in vivo* with a polynucleotide vector or plasmid of the invention. In one embodiment, the polynucleotide vector can be delivered to the kidney via catheter inserted into the proximal tubule. The amount of vector to be administered can be readily determined by a person of ordinary skill in the art.

HO-1 polynucleotide sequences included within the scope of the present invention include those sequences that encode HO-1 polypeptide that are derived from animals, including mammals. Preferably, the sequence is a human sequence of HO-1.

HO-1 polynucleotide sequences contemplated in the subject invention also include HO-1 genes having the natural sequence, as well as allelic variants and degenerate variants that encode an enzyme having HO-1 activity. The subject invention also concerns polynucleotide vectors and plasmids comprising polynucleotide sequences that encode a fragment or variant of an active HO-1 protein as long as that fragment or variant has substantially the same biological activity as the natural full length protein. Such fragments and variants can be readily prepared using standard methods. Thus, contemplated in all aspects of the invention are HO-1 polypeptides, and the polynucleotide sequences that encode them, as well as biologically active fragments and variants. The vectors of the subject invention can also be designed for targeted delivery and/or expression of the HO-1 gene in kidney cells. Targeted expression can be achieved by using suitable polynucleotide regulatory control elements to control expression of vector linked genes in particular cells or types of cells.

The subject invention also concerns polynucleotide expression vectors and plasmids comprising a polynucleotide sequence that encodes a heme oxygenase-1 enzyme, or a biologically active fragment or variant thereof. An exemplified polynucleotide plasmid of the subject invention has been designated pcDNA3.1/HO-1 (Figure 1A). This plasmid was prepared by inserting an EcoRI/Xbal fragment that includes the HO-1 protein coding region into the pcDNA3.1/zeo plasmid. This vector can be prepared by using standard procedures well known in the art. It comprises a cytomegalovirus promoter/enhancer and the protein coding region of the HO-1 gene. The procedure disclosed in Abraham *et al.* (Abraham, Nader G., Jean-Louis da Silva,

Yan Lavrovsky, Robert A. Stolz, Attallah Kappas, Michael W. Dunn, Michael Laniado Schwartzman (1995) "Adenovirus-Mediated Heme Oxygenase-1 Gene Transfer Into Rabbit Ocular Tissues" *Investigative Ophthalmology & Visual Science* 36(11):2202-2210) can be used in constructing suitable vectors of the subject invention by substituting the well-known cytomegalovirus for the adenovirus described in the publication.

Preferably, the polynucleotide vectors contain regulatory elements that provide for high levels of expression of an HO-1 encoding polynucleotide in the cells. More preferably, the polynucleotide vectors include promoter and/or enhancer elements selected for high levels of expression of any operably linked genes, such as HO-1, in mammalian cells. In an exemplified embodiment, the polynucleotide vector comprises cytomegalovirus promoter and/or enhancer sequences operably linked to that region of the HO-1 gene that encodes an active form of the enzyme. Other promoter sequences, such as adeno-associated virus inverted terminal repeat sequences as described in published international application number PCT/US93/05310, are contemplated for use with the subject invention. Preferably, the polynucleotide vector also comprises a polynucleotide encoding selectable marker, such as Zeocin, neoR, thymidine kinase, beta-galactosidase, chloroamphenicol acetyl transferase (CAT), dihydrofolate reductase (DHFR) and the like, which allows one to select for those cells that are stably transformed by the polynucleotide vector. The vector can also comprise a reporter gene for detection of HO-1 expression.

The polynucleotide sequences, including polynucleotide vectors and plasmids, of the subject invention can be composed of either DNA or RNA. Nucleotide analogs that can replace the normal nucleotides found in DNA and RNA can also be used in the subject polynucleotides, vectors, and plasmids.

The subject invention also concerns cells and tissues transformed with a polynucleotide expression vector comprising a polynucleotide sequence which encodes a heme oxygenase-1 enzyme. Transformed cells in tissues contemplated within the scope of the invention include kidney, spinal cord, brain, liver, lung, intestine and skin. Kidney cells that can be transformed according to the subject invention include, for example, human proximal tubule cells.

The subject invention also concerns polynucleotides comprising a first polynucleotide sequence that encodes an HO-1 protein, or a biologically active fragment

or variant thereof, in combination with a second polynucleotide sequence that encodes a protein having anti-oxidant activity. Examples of anti-oxidant proteins that can be encoded by the second polynucleotide and that are contemplated within the scope of the invention include superoxide dismutase (SOD) polypeptides. In a preferred embodiment, the second polynucleotide sequence encodes a mutant manganese SOD protein that lacks or that has decreased product inhibition as compared to wild type SOD. In another preferred embodiment, the polynucleotide encoding the SOD is operatively linked to a polynucleotide leader sequence that targets the polynucleotide to mitochondria. Most preferably, the second polynucleotide sequence encodes a mutant manganese SOD protein that lacks or that has decreased product inhibition as compared to wild type SOD and is operatively linked to a polynucleotide leader sequence that targets the polynucleotide to mitochondria The first and second polynucleotides can be operatively linked on a single vector or they can be present on separate vectors. Preferably, the first and second polynucleotides are operatively linked on a single vector. The order of the first and second polynucleotides on the vector is unimportant as long as proper protein expression from each of the protein encoding polynucleotides can be achieved once the vector is delivered to a target cell. In a preferred embodiment, the first and second polynucleotides are operatively linked on a single vector wherein an internal ribosome entry site (IRES) (Rees, S., Coote, J., Stables, S., Harris, S. and Lee, M.G., (1996) "Bicistronic vector for the creation of stable mammalian cell lines that predisposes all antibiotic-resistant cells to express recombinant protein" *Biotechniques* 20:102-110; CLONTECH 1998/99 catalog, Palo Alto, CA) sequence is provided between them. The polynucleotide vectors of the invention comprising first and/or second polynucleotides can further comprise regulatory sequences that enhance or promote expression of the polynucleotide sequences encoding the polypeptides. Preferably, the regulatory sequences are sequences that can be induced to upregulate expression of the encoded proteins in the target cell. More preferably, the regulatory sequences upregulate expression of the encoded proteins in the target cell in response to a physiological phenomena, such as, for example, an inflammatory or immune response in the host animal. Suitable regulatory sequences include promoter sequences, enhancer sequences, and intron sequences.

The present invention also concerns methods for regulating or inhibiting cellular

apoptosis. In one embodiment, apoptosis is regulated or inhibited in a target cell by expression of a polynucleotide sequence that encodes an HO-1 polypeptide. In a preferred embodiment of the subject invention, a polynucleotide encoding an HO-1 polypeptide is delivered to and expressed in a target cell. In another embodiment, the expression of an endogenous polynucleotide encoding an HO-1 polypeptide in a target cell is induced by administering an agent capable of inducing endogenous HO-1 expression. As shown in Figure 7, pre-treatment of human proximal tubule cells with hemin to induce HO-1 expression protects cells against cisplatin mediated apoptosis as measured by the Annexin I assay.

The present invention also concerns methods for enhancing transplantation success of tissues and organs in xenografts and allografts. In one embodiment, transplantation success of a xenograft or allograft in a host animal is enhanced by expression of a polynucleotide sequence that encodes an HO-1 polypeptide. In a preferred embodiment of the subject invention, a polynucleotide encoding an HO-1 polypeptide is delivered to and expressed in a target cell of the transplanted tissue or organ. In another embodiment, the expression of an endogenous polynucleotide encoding an HO-1 polypeptide in a target cell of the transplanted tissue or organ is induced by administering an agent capable of inducing endogenous HO-1 expression. Tissue and organs contemplated for transplantation include, but are not limited to, heart, lung, liver, kidney, skin, spinal cord and fetal tissues.

The present invention also concerns methods for treating or preventing conditions such as atherosclerosis, acute respiratory distress conditions, ischemia-reperfusion injury, radiation-induced injury and other conditions that involve oxidative stress. The subject invention also concerns methods for treating cadmium exposure which causes nephrotoxicity. In one embodiment, a target cell is treated or induced to express a polynucleotide sequence that encodes an HO-1 polypeptide. In a preferred embodiment of the subject invention, a polynucleotide encoding an HO-1 polypeptide is delivered to and expressed in a target cell. In another embodiment, the expression of an endogenous polynucleotide encoding an HO-1 polypeptide in a target cell is induced by administering an agent capable of inducing endogenous HO-1 expression.

The subject invention also pertains to polynucleotides having sequences that are antisense to polynucleotides that encode HO-1, or a fragment or variant thereof. The

antisense polynucleotides include those that are antisense to both DNA encoding HO-1, as well as RNA transcribed from that DNA. The antisense sequences of the subject invention can be delivered to and expressed in target cells, such as cancer cells, for which one would like to decrease or prevent expression of HO-1. Thus, the subject invention also concerns methods for cancer therapy by inhibiting expression of HO-1 in targeted cancer cells using the antisense sequences of the invention.

All publications cited herein are hereby incorporated by reference.

Materials and Methods

Transfection:

Cells were transfected using the lipofectamine method (Life Technologies, Gaithersburg, MD).

LDH assay:

Transfected HEK 293 cells were plated in 24 well plates and incubated at 37°C for 24 hours. The cells were exposed to CP(100μM). Sixteen hours after exposure, 100μl of supernatant was transferred in 96 wll plates and 200 μl of working solution containing diaphorase, NAD^+, iodo-tetrazolium chloride and sodium lactate were added. The enzyme reaction was stopped by the addition of 50 μl of 1N HCl. The absorbance of samples at 492 nm and 620 nm was measured. The percent specific LDH release was calculated by the absorbance of treated samples over control and Triton-X treated samples.

Assessment of mitochondrial viability by the MTT assay:

Transfected cells were plated into 96 well plates and incubated for 3 days, followed by exposure to media containing PBS (control) or CP (100μM) for 16 hours. Ten microliters of 5 mg/ml MTT stock solution was added and cells were incubated for 4 hours. One hundred μl of acidic isopropanol (0.04N HCl in isopropanol) was added. The absorbance of samples at 550nm and 690nm was measured. Results are expressed as percentage change in absorbance over control cells in each group.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example - 1

In order to show that cells transfected with the polynucleotide expression vector of the subject invention provided for increased expression of the HO-1 gene, approximately 300 base pairs of nucleotides were removed from the 3' prime noncoding sequence of the HO-1 cDNA on the vector. Because the heterologous HO-1 mRNA will be shorter in size than an endogenous HO-1 mRNA, it was possible to distinguish expression of endogeneous HO-1 mRNA from heterologous HO-1 mRNA that is transcribed from the subject vector in the cells. Figure 1B shows a Northern blot, using the pcDNA3.1/Zeo plasmid as a probe, of RNA from both untreated human renal tubule cells (lane 1) and human renal tubule cells transformed with an expression vector having the shorter HO-1 cDNA according to the subject invention (lane 2). As can be seen in lane 1, the untreated cells express only endogenous HO-1 mRNA. Lane 2 of Figure 1B shows that the transformed cells express the endogenous HO-1 mRNA and overexpress the shorter HO-1 mRNA transcribed from the subject vector.

Example - 2

Human proximal tubule cells were transfected with either a vector lacking the HO-1 gene or with vector that included a polynucleotide sequence encoding the HO-1 enzyme. Cells were then exposed to varying concentrations of cisplatin (10 μM, 50 μM, and 100 μM). Lactate dehydrogenase (LDH) release was then measured from the cells. Release of lactate dehydrogenase from cells reflects the damage to the cells as a result of cytotoxicity of cisplatin. Thus, the greater percentage release of LDH from cells correlates with greater cytotoxicity associated with cisplatin treatment. Figure 2 shows that cells expressing the HO-1 gene exhibited an approximately 30% decrease in LDH release at both the 50 μM and 100 μM concentrations of cisplatin. Thus, overexpression of HO-1 in these cells provided a cytoprotective effect against damage from cisplatin as measured by LDH release from the cells.

Example - 3

Figure 3 shows the morphology of renal tubule cells either untreated (Panel A) or treated with cisplatin (Panel B) or pretreated with hemin (an inducer of HO-1 expression) followed by cisplatin exposure (Panel C). As can be seen in the micrographs, cells exposed to cisplatin alone show a damaged morphology. In contrast, cells pretreated with hemin and then exposed to cisplatin retain morphology and cell structure strikingly similar to untreated control cells. Thus, this provides evidence that expression of HO-1 enzyme in these cells can confer a cytoprotective effect from damage due to treatment with cisplatin and other agents which cause heme related damage to cells.

Example - 4

As shown in Figure 6, the cytoprotective role of prior induction of HO-1 in cytotoxicity induced by cisplatin (CP) in human proximal tubule cells (HPTC) was evaluated. Confluent HPTC were pretreated for 2h with low dose hemin (5μM), a potent inducer of HO-1, 12h prior to exposure to CP (100μM) for 12-24h. Cytotoxicity was determined by the LDH assay and expressed as % LDH release over control. A 6- and 4-fold increase in HO-1 protein expression occurred at 12 and 24h, respectively, in response to hemin treatment. Prior induction of HO-1, by hemin pretreatment, significantly attenuated CP-induced cytotoxicity by 60% (CP alone: 16.1±2.7%; hemin+CP; 6.35±1.6%; $p<0.05$)($n=4$) at 12h. The specificity of the cytoprotective effect of HO-1 was confirmed by co-treatment of cells with zinc protoporphyrin (ZnPP) (5μM), an inhibitor of HO-1. Inhibition of HO-1 with ZnPP resulted in reversal of the cytoprotective effect conferred by hemin pretreatment (hemin+CP+ZnPP: 15.4±1.8%). Hemin and ZnPP alone demonstrated no significant cytotoxicity. The protective effect of hemin pretreatment was also seen after 24h of exposure to CP (100μM) (CP:36.5%; Hemin+CP: 17.6%, $p<0.05$).

These data demonstrate a protective role of prior induction of HO-1 in CP-induced cytotoxicity in HPTC. Measures targeted to overexpress HO-1 may have implications in designing possible therapeutic approaches to attenuate CP nephrotoxicity while still taking advantage of the beneficial antineoplastic properties of CP as a treatment for cancer.

Example - 5

The beneficial effects of the anticancer drug, cisplatin, are mitigated by significant nephrotoxicity. Studies were performed to manipulate the HO-1 gene at the molecular level resulting in selective overexpression in our model of cisplatin-induced cytotoxicity. HEK293 cells were transfected with a vector containing the entire protein coding region of the human HO-1 gene (HHO) under the promoter/enhancer of cytomegalovirus (pcDNA3.1/HHO). The vector alone was used as a transfection control. Overexpression was confirmed by northern and immunoblot analyses which revealed a ~40 fold and ~10 fold increase in mRNA and protein, respectively, over control cells (Figure 1B and 1C). Cells were exposed to cisplatin (100μM) for 16 hours and cytotoxicity assessed by phase contrast microscopy, LDH assay and the MTT assay. Morphological indices of injury such as cell rounding, vacuolization, and detachment were significantly reduced in cells with elevated levels of HO-1 compared to cells transfected with the vector alone. These changes were accompanied by a significant attenuation of cisplatin-induced cytotoxicity by 68% in such cells as determined by LDH release (Vector alone: 20+/-6.8%, HO-1 overexpression: 6.6+/-2.82, $p<0.05$, n=4) (see Figure 5). Protection from cell injury was also confirmed by the MTT assay. This assay requires the presence of active mitochondrial dehydrogenases to reduce the dye MTT to formazan, a purple colored dye. Exposure to cisplatin (100μM) for 16 hours resulted in significant impairment in the ability of cells transfected with vector alone to reduce MTT compared to HO-1 overexpressing cells (Vector alone, 16.6+/-2.8 vs. HO-1 overexpression, 5.26+/-2.3%; $p<0.05$, n=3) (Figure 8). Results are expressed as percentage change in MTT over control untreated cells in each group. Treatment of cells with a competitive inhibitor, zinc protoporphyrin, reversed the cytoprotective effects of HO-1. These data demonstrate the protective role of HO-1 in cisplatin nephrotoxicity and in high risk settings of acute renal failure.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

1. A polynucleotide expression vector comprising a polynucleotide sequence encoding a heme oxygenase -1 (HO-1) enzyme, or a biologically active fragment or variant thereof.

2. The polynucleotide expression vector according to claim 1, wherein said vector comprises the amino acid coding region of the polynucleotide sequence shown in Figure 4B.

3. The polynucleotide expression vector according to claim 1, wherein said vector comprises regulatory sequences operably linked with said polynucleotide sequence encoding said heme oxygenase-1 enzyme, wherein said regulatory sequences promote expression of said polynucleotide sequence.

4. The polynucleotide expression vector according to claim 3, wherein said regulatory sequence comprises a promoter or enhancer sequence.

5. The polynucleotide expression vector according to claim 4, wherein said promoter or enhancer sequence is derived from cytomegalovirus.

6. The polynucleotide expression vector according to claim 1, wherein said vector is derived from a viral vector.

7. The polynucleotide expression vector according to claim 6, wherein said viral vector is selected from the group consisting of adeno-associated virus, retrovirus, and adenovirus.

8. The polynucleotide expression vector according to claim 1, wherein said vector comprises a polynucleotide sequence encoding a heme oxygenase-1 enzyme comprising the amino acid sequence shown in Figure 4A.

9. A method for protecting cells or tissue from damage, said method comprising transforming said cells or tissue with the polynucleotide expression vector of claim 1 to express a heme oxygenase-1 enzyme in said cells or tissue.

10. A cell transformed with the polynucleotide expression vector of claim 1.

11. The cell of claim 10, wherein said cell is a kidney cell.

12. The cell of claim 11, wherein said kidney cell is a human renal proximal tubal cell.

13. A tissue transformed with the polynucleotide expression vector of claim 1.

14. The tissue of claim 13, wherein said tissue is from kidney.

15. A method for treating a patient, wherein said patient is to receive or is receiving treatments with agents that cause or are associated with heme protein-related damage or oxidative-related damage to cells or tissues in said patient, said method comprising transforming cells or tissue with a polynucleotide, wherein expression of said polynucleotide provides a heme oxygenase-1 enzyme in said cells or tissue.

16. The method according to claim 15, wherein said agent is an agent for treating a condition selected from the group consisting of infection, cancer, and immunosuppression.

17. The method according to claim 15, wherein said agent is a radiocontrast agent.

18. The method according to claim 15, wherein said cell is selected from the group consisting of kidney cell, brain cell, liver cell, lung cell, skin cell, gastrointestinal cell, and spinal cord cell.

19. The method according to claim 15, wherein said heme oxygenase-1 enzyme is overexpressed in said cell or tissue relative to a cell or tissue that has not been transformed with said polynucleotide.

20. The method according to claim 15, wherein said polynucleotide is provided in the form of an expression vector.

21. The method according to claim 20, wherein said expression vector comprises a suitable promoter sequence to promote expression of said heme oxygenase-1 enzyme in said cell or tissue.

22. The method according to claim 21, wherein said promoter is selected from the group consisting of cytomegalovirus promoters, adenovirus promoters and adeno-associated virus promoters.

23. The method according to claim 20, wherein said expression vector comprises an enhancer sequence.

24. The method according to claim 20, wherein said expression vector is selected from the group consisting of retrovirus vectors, adeno-associated virus vectors and adenovirus vectors.

25. The method according to claim 15, wherein said cell or tissue is transformed *in vivo* with said polynucleotide.

26. The method according to claim 15, wherein said cell or tissue is transformed *ex vivo* with said polynucleotide.

27. The method according to claim 20, wherein said expression vector is pcDNA 3.1/Zeo.

28. A polynucleotide molecule, wherein said polynucleotide comprises a first polynucleotide sequence encoding a heme oxygenase-1 protein, or a biologically active fragment or variant thereof, and a second polynucleotide sequence encoding a protein having antioxidant activity.

29. The polynucleotide according to claim 28, wherein said second polynucleotide sequence encodes a superoxide dismutase protein.

30. The polynucleotide according to claim 29, wherein said superoxide dismutase protein encoded by said second polynucleotide is a mutant superoxide dismutase protein that exhibits decreased product inhibition as compared to wild-type superoxide dismutase protein.

31. The polynucleotide according to claim 28, wherein said second polynucleotide sequence encoding said superoxide dismutase protein is operatively linked to a polynucleotide leader sequence that targets said polynucleotide to mitochondria.

32. The polynucleotide according to claim 28, wherein an internal ribosome entry site sequence is provided between said first and second polynucleotide sequences.

33. The polynucleotide according to claim 28, wherein said polynucleotide comprises a regulatory sequence that enhances or promotes expression of said first or second polynucleotide sequences.

34. The polynucleotide according to claim 33, wherein said regulatory sequence upregulates expression of said first or second polynucleotide sequences in response to a physiological response.

35. The polynucleotide according to claim 34, wherein said physiological response is selected from the group consisting of an inflammatory response and an immune response.

36. The polynucleotide according to claim 33, wherein said regulatory sequences are selected from the group consisting of promoter sequences, enhancer sequences and intron sequences.

37. A polynucleotide composition, wherein said polynucleotide composition comprises a first polynucleotide molecule encoding a heme oxygenase-1 protein, or a biologically active fragment or variant thereof; and a second polynucleotide molecule comprising a polynucleotide sequence encoding a protein having antioxidant activity.

38. A method for regulating or inhibiting cellular apoptosis, said method comprising transforming a cell with the polynucleotide expression vector of claim 1 to express a heme oxygenase-1 enzyme in said cell.

Fig.1: Plasmid construction and verification of HO-1 overexpression

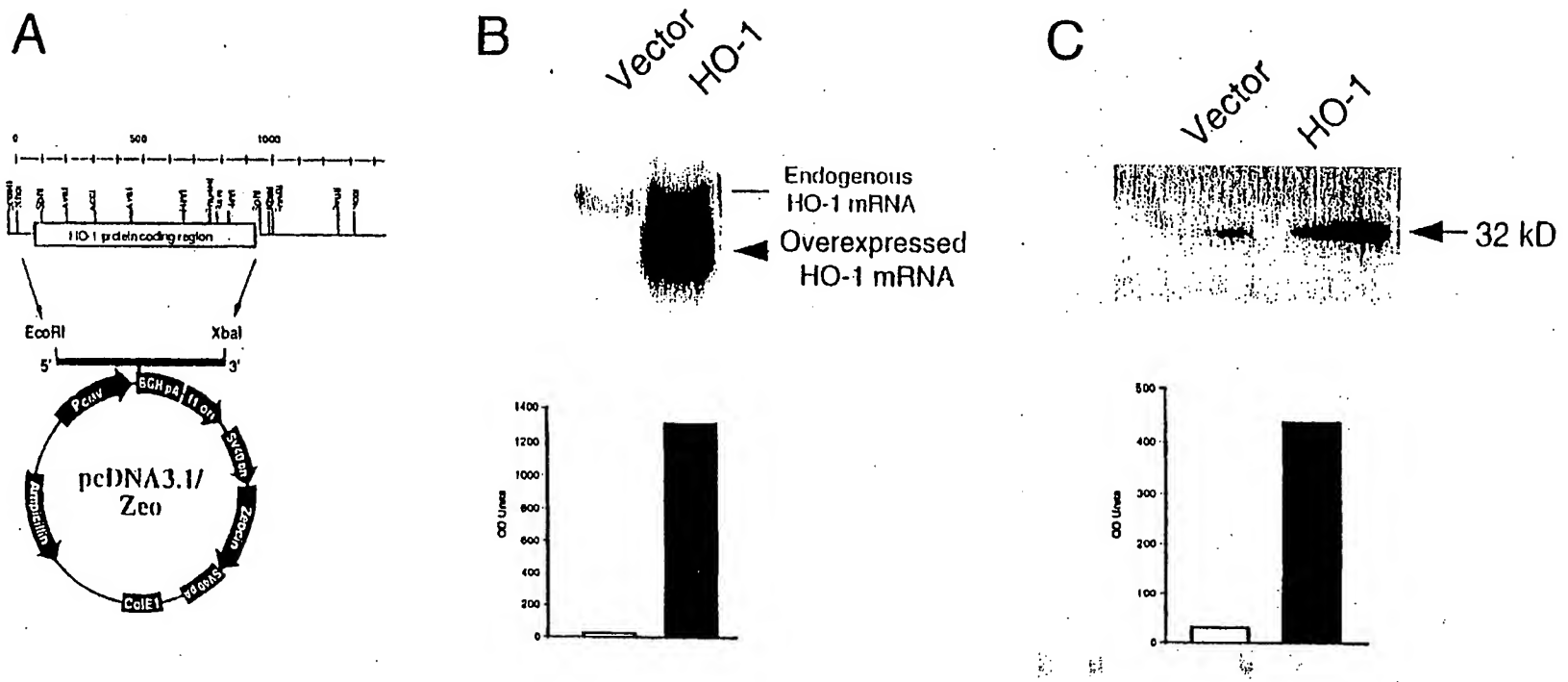

FIG. 2

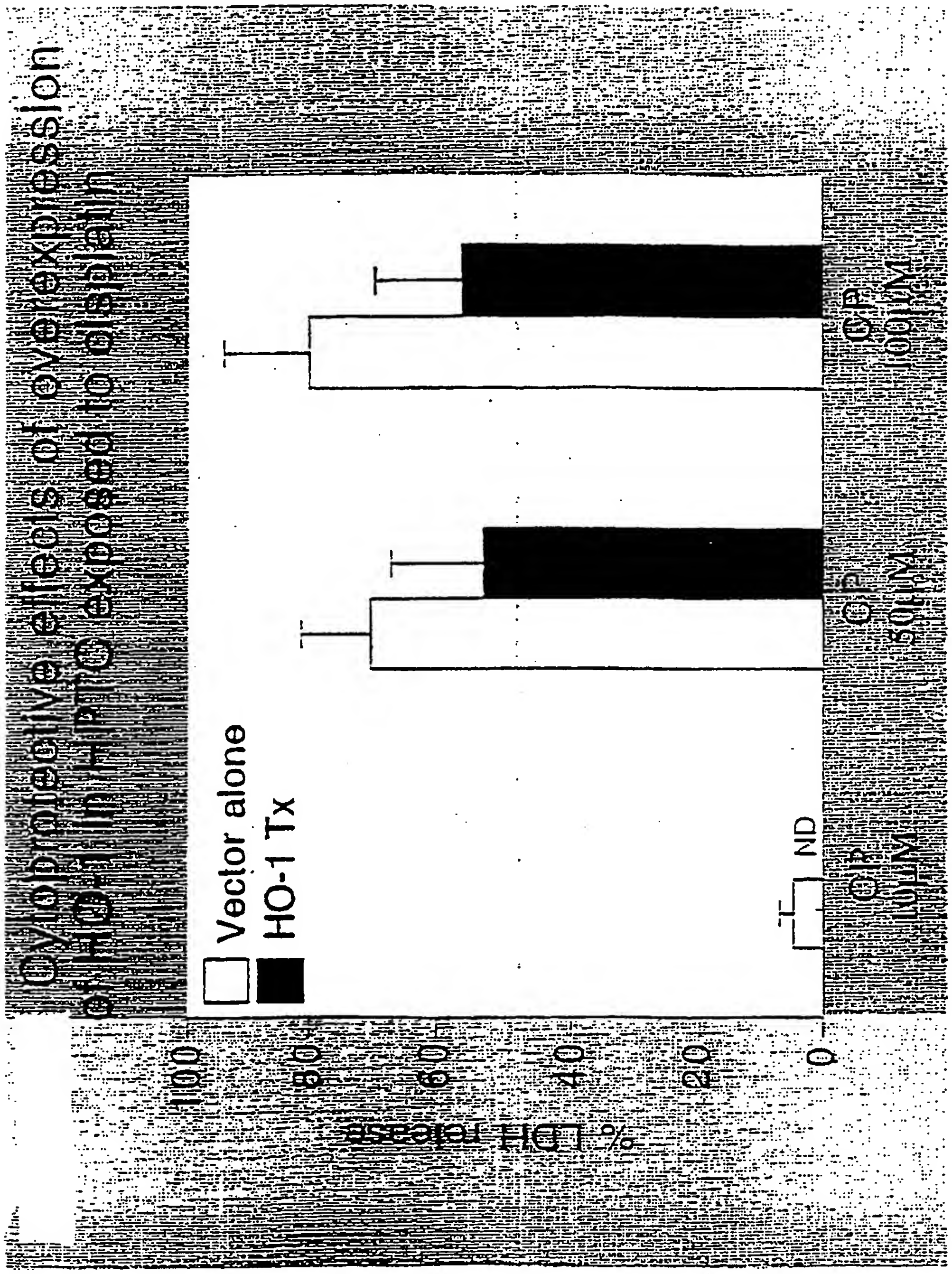
Cytoprotective effects of overexpression of HO-1 in HPTC exposed to cisplatin
Vector alone
HO-1 Tx
% LDH release
100
80
60
40
20
0
ND
CP 10 µM
CP 50 µM
CP 100 µM

FIG. 3A

Control

FIG. 3B

Cisplatin 100μM

FIG. 3C

Hemin pretreated + Cisplatin 100μM

/translation="MERPQPDSMPQDLSEALKEAIKEVETQAENAEFMRNFQKGQVIR
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LVATVAVGLYAM"

FIG 4A

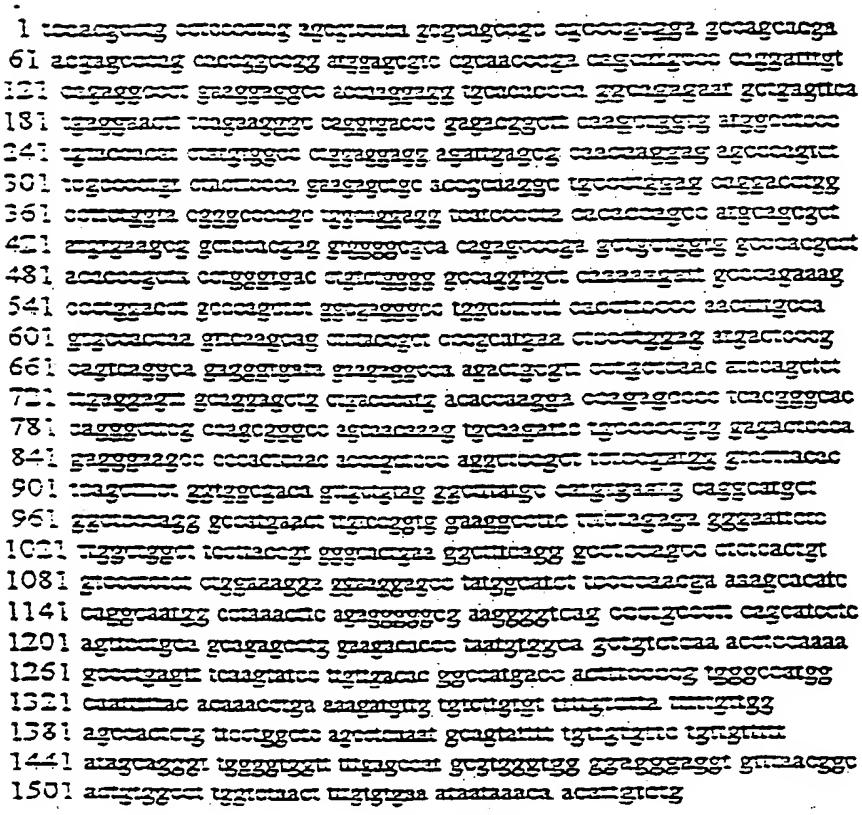


FIG. 4B

Fig 5: Cytoprotective effects of HO-1 overexpression in HEK 293 cells exposed to cisplatin

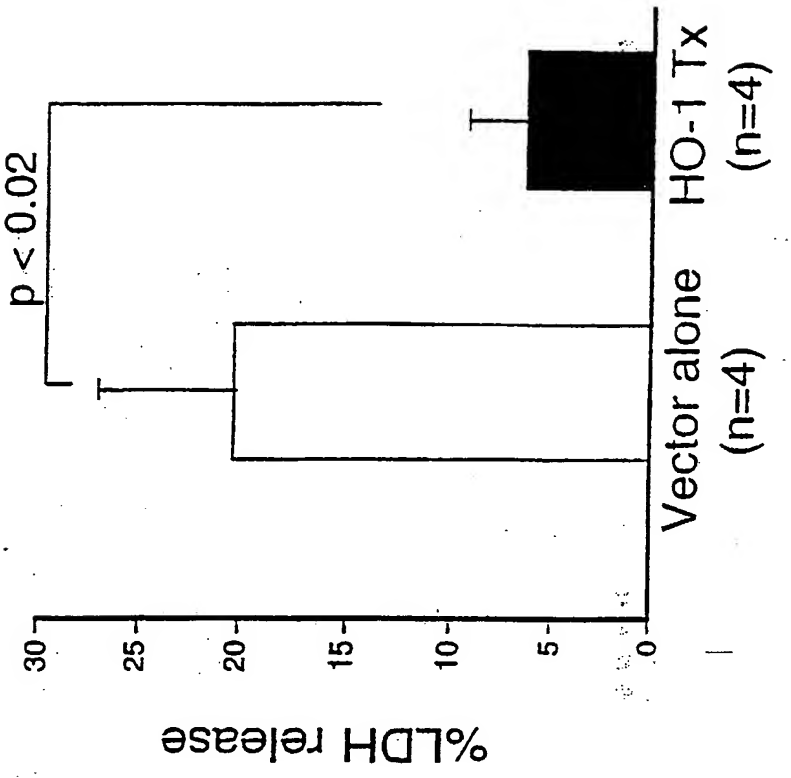
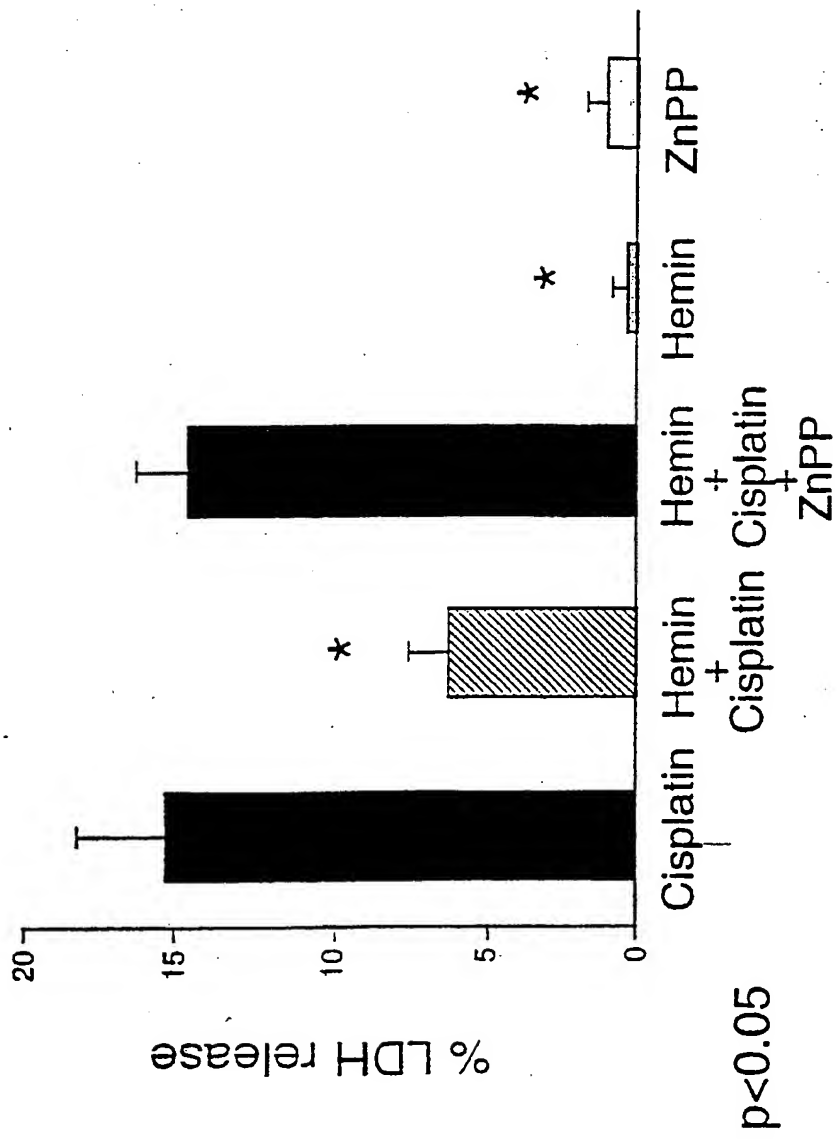
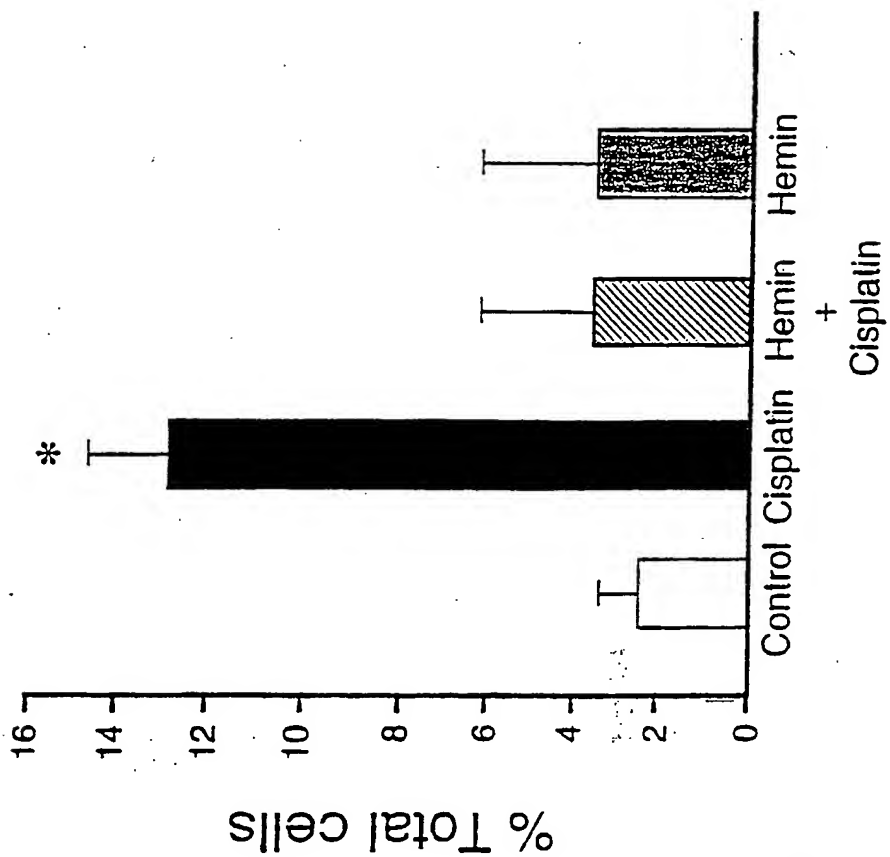


Fig. 6: Cytoprotective effect of Hemin pretreatment in proximal tubule cells exposed to Cisplatin



* $p<0.05$

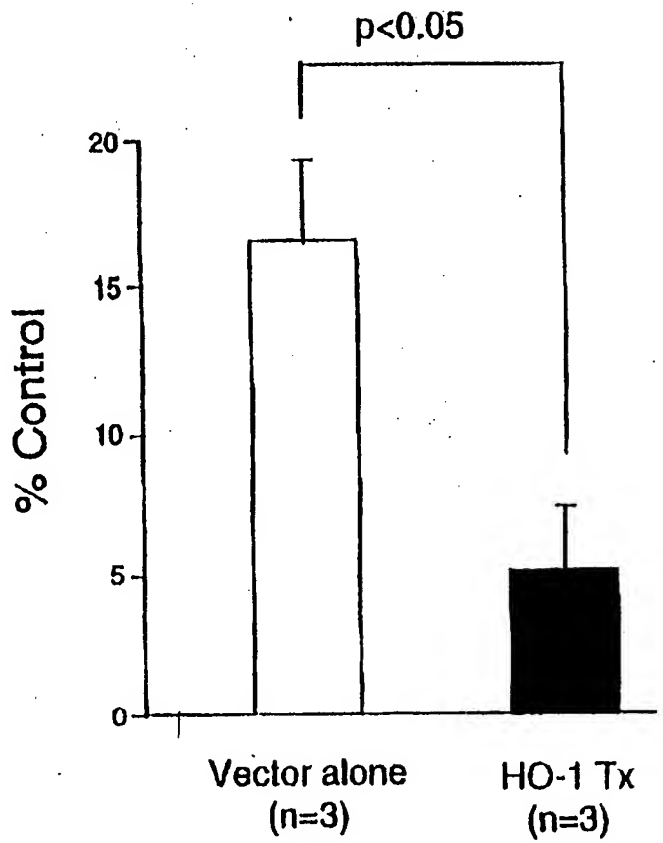
Fig. 7: Effect of hemin pretreatment on Cisplatin-induced apoptosis in human proximal tubule cells



* $p<0.05$

Mitochondrial viability: MTT assay

Fig. 8 Cytoprotective effects of overexpression of HO-1 in HEK 293 cells exposed to cisplatin (100μM)



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant: UNIVERSITY OF FLORIDA [US/US]; 223 Grinter Hall, Gainesville, FL 32611 (US).

(72) Inventors: AGARWAL, Anupam; 4015 N.W. 59th Avenue, Gainesville, FL 32653 (US). NICK, Harry, S.; 6324 N.W. 97th Court, Gainesville, FL 32653 (US). VISNER, Gary, A.; R.R. 2, Box 430–B, Alachua, FL 32615 (US).

(74) Agents: PACE, Doran, R. et al.; Saliwanchik, Lloyd & Saliwanchik, P.A., Suite A–1, 2421 N.W. 41st Street, Gainesville, FL 32606–6669 (US).

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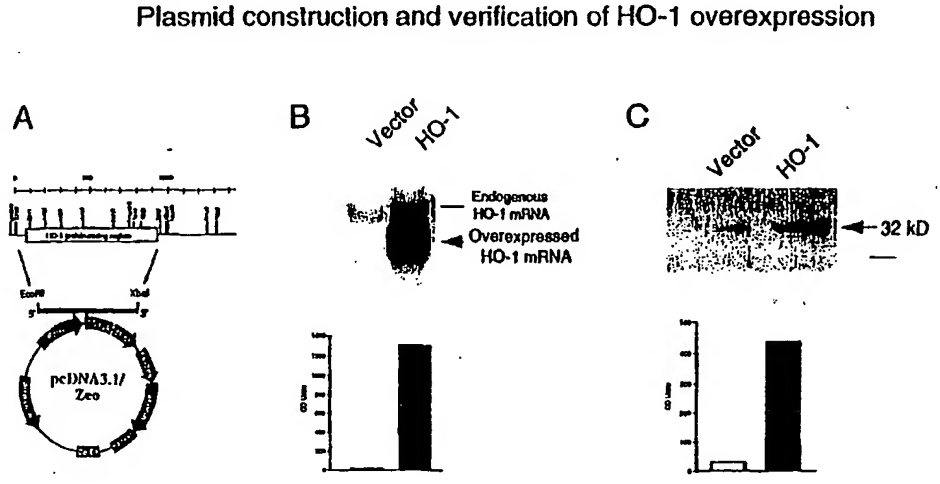

(57) Abstract

The subject invention concerns a novel process for treating a human or animal to prevent cellular or tissue injury when said human or animal is at risk for heme protein–related damage or injury, such as acute renal failure that is associated with cisplatin treatment of cancer patients. The process employs a novel vector which, via *in vivo* gene transfer, results in the overexpression of the HO–1 gene in the kidney. The subject invention also concerns polynucleotide vectors and plasmids comprising the polynucleotide sequences that encode an HO–1 enzyme. These vectors can be used for transforming kidney cells to express the HO–1 gene.

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INTERNATIONAL SEARCH REPORT

Interna l Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 C12N15/86 C12N15/62 C12N5/10 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABRAHAM, N.G. ET AL.: "Adenovirus-mediated heme oxygenase-1 gene transfer into rabbit ocular tissues" INVESTIGATIVE OPHTHALMOLOGY & VISUAL SCIENCE, vol. 36, no. 11, October 1995, pages 2202-2210, XP002086227 cited in the application	1-10,13
Y	see the whole document	11,12, 14-26, 28,29, 32-37

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 April 1999	07. 05. 99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Donath, C

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INTERNATIONAL SEARCH REPORT

Intern. al Application No
PCT/US 98/23270

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABRAHAM, N.G. ET AL.: "Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: protective effect against heme and hemoglobin toxicity" PROC.NATL.ACAD.SCI.USA, vol. 92, no. 15, 18 July 1995, pages 6798-6802, XP002100374	1-5,8, 10,13
Y	see page 6798 - page 6801 'Materials and Methods' and 'Results' ---	15-23, 25,26, 28,29, 32-37
X	LEE, P.J. ET AL.: "Overexpression of heme oxygenase-1 in human pulmonary epithelial cells results in cell growth arrest and increased resistance to hyperoxia" PROC.NATL.ACAD.SCI.USA, vol. 93, September 1996, pages 10393-10398, XP002100375 cited in the application see page 10393 - page 10396 'Materials and Methods' and 'Results' ---	1,3,4,9, 10
Y	AGARWAL, A. ET AL.: "Induction of heme oxygenase in toxic renal injury: A protective role in cisplatin nephrotoxicity in the rat " KIDNEY INTERNATIONAL, vol. 48, no. 4, October 1995, pages 1298-1307, XP002100376 cited in the application see the whole document ---	11,12, 14-26
X	DENNERY, P.A. ET AL.: "Heme oxygenase-mediated resistance to oxygen toxicity in hamster fibroblasts" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 23, 6 June 1997, pages 14937-14942, XP002100598	1-5,8, 10,13
Y	see page 14939 - page 14942 'Results and Discussion' ---	15-23, 25,26, 28,29, 32-37
Y	WO 92 07935 A (THE SCRIPPS RESEARCH INSTITUTE) 14 May 1992 see page 6, line 26 - page 7, line 22 see page 23, line 21 - page 26, line 11 see page 55, line 5 - page 59, line 4 see page 66, line 13 - page 70, line 1 --- -/--	28,29, 32-37

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INTERNATIONAL SEARCH REPORT

Intern. al Application No
PCT/US 98/23270

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YOSHIDA, T. ET AL.: "Human heme oxygenase cDNA and induction of its mRNA by hemin" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 171, no. 3, 1 February 1988, pages 457-461, XP002100377 cited in the application see figure 4 -----	2,8

1

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INTERNATIONAL SEARCH REPORT

Inte. .tional application No.
PCT/US 98/23270

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 15-25, 27 and 38 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. ...al Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9207935 A	14-05-1992	AU 8947791 A	26-05-1992

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A3

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(71) Applicant: UNIVERSITY OF FLORIDA [US/US]; 223 Grinter Hall, Gainesville, FL 32611 (US).

(72) Inventors: AGARWAL, Anupam; 4015 N.W. 59th Avenue, Gainesville, FL 32653 (US). NICK, Harry, S.; 6324 N.W. 97th Court, Gainesville, FL 32653 (US). VISNER, Gary, A.; R.R. 2, Box 430–B, Alachua, FL 32615 (US).

(74) Agents: PACE, Doran, R. et al.; Saliwanchik, Lloyd & Saliwanchik, P.A., Suite A–1, 2421 N.W. 41st Street, Gainesville, FL 32606–6669 (US).

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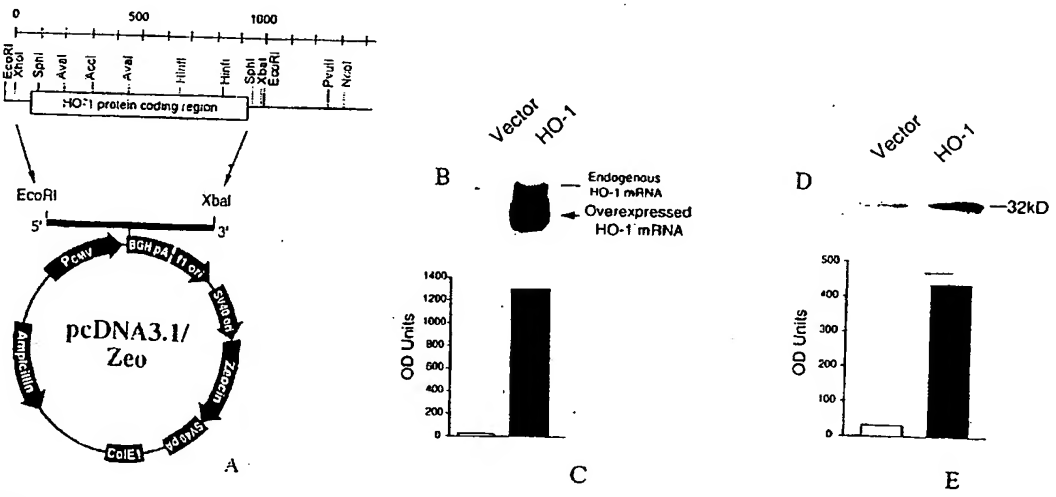

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BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

DESCRIPTION

MATERIALS AND METHODS FOR PREVENTING CELLULAR INJURY IN HUMANS AND ANIMALS

This invention was made with government support under United States National Institutes of Health Grant No. NIDDK K08 DK02446-01. The government has certain rights in this invention.

Cross-Reference to Related Applications

This application claims the benefit of U.S. Provisional Application Serial No. 60/063,863, filed October 31, 1997 and U.S. Provisional Application Serial No. 60/105,400, filed October 23, 1998.

Background of the Invention

Damage to organs, tissues and cells can arise when heme proteins, such as hemoglobin and myoglobin, escape from the intracellular space. Heme oxygenase - 1(HO-1) is a 32 kDa microsomal enzyme that is important in the degradation of heme. HO-1 catalyzes the conversion of heme to biliverdin, releasing iron and carbon monoxide. Biliverdin is subsequently converted to bilirubin which is then excreted from the body. HO-1 expression is inducible not only by the heme substrate, but also by a variety of agents that are associated with, or that cause oxidative stress. A human HO-1 cDNA nucleotide sequence, and the amino acid sequence encoded thereby, has been determined (Yoshida, T., P. Biro, T. Cohen, R.M. Muller, and S. Shibahara (1988) "Human heme oxygenase cDNA and induction of its mRNA by hemin" *Eur. J. Biochem.* **171**(3):457-461; Shibahara, S., M. Sato, R.M, Muller, T. Yoshida. (1989) "Structural organization of the human heme oxygenase gene and the function of its promoter" *Eur. J. Biochem.* **179**: 557-563; Takeda, K., S. Ishizawa, M. Sato, T. Yoshida, S. Shibahara. (1994) "Identification of a cis-acting element that is responsible for cadmium-mediated induction of the human heme oxygenase gene" *J.Biol. Chem.* **269**: 22858-22867).

The literature suggests that the induction of heme oxygenase-1 is an adaptive and

protective response to a wide variety of stimuli in cells and tissues. Most of these studies utilized chemical inducers and inhibitors of HO-1 to establish a functional role for HO-1 in cytoprotection. Previous studies have demonstrated that chemical inducers of the HO-1 gene demonstrate significant protective responses, while inhibition of the HO-1 gene with chemical inhibitors worsen cell injury both *in vivo* and *in vitro* (Agarwal, Anupam, József Balla, Jawed Alam, Anthony J. Croatt, Karl A. Nath (1995) "Induction of heme oxygenase in toxic renal injury: A protective role in cisplatin nephrotoxicity in the rat" *Kidney International* **48**:1298-1307; Shiraishi, Furnie, Harry S. Nick, C. Craig Tisher, Agarwal, Anupam, (1997) Abstract - 30th Annual Meeting (November 2-5, 1997) "Prior Induction Of Heme Oxygenase-1 Attenuates Cisplatin-Mediated Toxicity In Human Proximal Tubule Cells (HPTC)"; and Nath, Karl A., Gyorgy Balla, Gregory M. Vercellotti, József Balla, Harry S. Jacob, Michael D. Levitt and Mark E. Rosenberg (1992) "Induction Of Heme Oxygenase is a Rapid, Protective Response in Rhabdomyolysis in the Rat" *J. Clin. Invest.* **90**:267-270). However, these chemicals may have nonspecific effects other than the induction of HO-1.

Studies have shown overexpression of HO-1 to be protective in toxicity against heme and hemoglobin (Abraham, N.G., Y. Lavrovsky, M.L. Schwartzman, R.A. Stoltz, R.D. Levere, M.E. Gerritsen, S. Shibahara, A. Kappas (1995) "Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: Protective effect against heme and hemoglobin toxicity" *Proc. Natl. Acad. Sci. USA* **92**:6798-6802), and hyperoxia (Lee, Patty J., Jawed Alam, Gordon W. Wiegand, Augustine M.K. Choi (1996) "Overexpression of heme oxygenase-1 in human pulmonary epithelial cells results in cell growth arrest and increased resistance to hyperoxia" *Proc. Natl. Acad. Sci. USA* 93:10393-10398). More recently, Abraham *et al.* (Abraham, Nader G., Jean-Louis da Silva, Yan Lavrovsky, Robert A Stoltz, Attallah Kappas, Michael W. Dunn and Michael Laniado Schwartzman (1995) "Adenovirus-Mediated Heme Oxygenase-1 Gene Transfer Into Rabbit Ocular Tissues" *Investigative Ophthalmology & Visual Science* **36**(11):2202-2210) have demonstrated a tissue-selective functional transfer of the human HO-1 gene into rabbit ocular tissues *in vivo*. Microinjection of an adenovirus-HHO construct mixed with lipofectamine into the eye resulted in HO-1 mRNA expression in different regions of the eye, without any expression in extraocular tissues such as the brain, liver or kidney. However, there are no studies in the literature that establish targeted gene

delivery of HO-1 to the kidney. Expression of heme oxygenase-1 has also recently been implicated in mouse cardiac xenograft survival in rats (Soares, M.P., Lin, Y., Anrather, J., Csizmdia, E., Takigami, K., Sato, K., Grey, S.T., Colvin, R.B., Choi, A.M., Poss, K.D., and Bach, F.H. (1998) "Expression of heme oxygenase-1 can determine cardiac xenograft survival" *Nature Medicine* 4:1073-1077).

Patients receiving medications for infection (*e.g.*, gentamicin), cancer (*e.g.*, cisplatin), immunosuppression (*e.g.*, cyclosporin), or radiocontrast agents have a high risk for sustaining damage to cells and tissues, such as, for example, damage to kidneys which can result in acute renal failure. Treatment for acute renal failure is expensive, necessitating dialysis and prolonged hospital stay. There is an increase in morbidity and mortality as well. Accordingly, there remains a need in the art for therapies that can prevent or ameliorate cell/tissue damage and/or failure associated with release of heme proteins.

Brief Summary of the Invention

The subject invention concerns methods for providing a person or animal with cytoprotection from heme protein-related cell damage and oxidative stress. Specifically, the subject method pertains to gene therapy using polynucleotide expression vectors or plasmids that include a nucleotide sequence encoding HO-1. Cells or tissue transformed with the subject vectors or plasmids overexpress HO-1 enzyme which catalyzes degradation of heme proteins.

The subject invention also concerns novel polynucleotide expression vectors and plasmids comprising a polynucleotide sequence encoding HO-1. In one embodiment, an expression vector comprising a cytomegalovirus promoter/enhancer and the protein coding region of the HO-1 gene (pcDNA3.1/HO-1) is exemplified. When transfected into target cells, vectors of the subject invention provide for overexpression of HO-1 in those cells.

The targeted expression system of the invention can be used as a therapeutic, and more importantly, as a preventative modality in treatments and procedures that present high risk for heme protein-related cytototoxicity or oxidative stress in a patient.

Brief Description of the Drawings

Figure 1A shows construction of a plasmid designated as pcDNA3.1/Zeo. **Figure 1B** shows a Northern blot of RNA from human renal tubule cells transfected with the pcDNA3.1/Zeo plasmid encoding the HO-1 enzyme. **Figure 1D** shows a Western blot of proteins from human renal tubule cells transfected with the pcDNA3.1/Zeo plasmid encoding the HO-1 enzyme. The Western blot confirms the presence of a 32 kD protein.

Figure 2 shows cytoprotective effects of transient overexpression of the HO-1 gene in human proximal tubule cells exposed to cisplatin.

Figures 3A, 3B, and 3C show micrographs of human renal tubule epithelial cells that are either (Figure 3A) untreated control, (Figure 3B) treated with 100 μM cisplatin, or (Figure 3C) pretreated with hemin and then challenged with 100μM cisplatin.

Figure 4A shows the amino acid sequence (in standard single letter code) of the human HO-1 enzyme. **Figure 4B** shows a cDNA sequence encoding the HO-1 enzyme.

Figure 5 shows overexpression of HO-1 decreases cisplatin mediated toxicity as measured by LDH assay.

Figure 6 shows cytoprotective effect of hemin pretreatment in proximal tubule cells exposed to cisplatin.

Figure 7 shows the effect of hemin pretreatment on cisplatin-induced apoptosis in human proximal tubule cells.

Figure 8 shows the effects of overexpression of HO-1 in HEK293 cells exposed to cisplatin as measured by mitochondrial viability by the MTT assay.

Detailed Disclosure of the Invention

The subject invention concerns methods and materials for protecting cells and tissues at risk for heme protein-related damage and/or exposure to oxidative stress. In one embodiment, a method of the subject invention comprises gene therapy of targeted cells or tissues to introduce polynucleotide sequences which when expressed result in overexpression of an enzyme having HO-1 activity. Cells and tissues contemplated for treatment by the subject methods include, for example, kidney, spinal cord, brain, liver, lung, intestine and skin.

Specifically contemplated within the scope of the present invention are methods

for treating cancer patients, transplant patients and others that are to receive treatments with injurious agents, such as cisplatinin and cyclosporine, that are associated with heme protein or oxidative-related damage to normal cells and tissues in the patient. In one embodiment, the subject method comprises utilizing gene therapy techniques to introduce polynucleotide sequences into targeted cells or tissues which when expressed in those cells or tissues result in overexpression of an HO-1 enzyme, or a biologically active fragment or variant thereof.

In one embodiment, the present invention concerns a method for providing overexpression of HO-1 protein to provide cytoprotection in human renal proximal tubule kidney cells. Cells in the proximal tubule of the nephron are most susceptible to a wide variety of injurious agents, *e.g.*, drugs (gentamicin, cisplatin, cyclosporine), contrast agents used in radiologic procedures, environmental toxins (cadmium) and ischemia-reperfusion renal injury. One embodiment of the subject method provides a polynucleotide expression vector comprising at least that region of an HO-1 gene that encodes an HO-1 enzyme, or a biologically active fragment or variant thereof, to transform kidney cells to express HO-1 in a patient in need of such treatment. Once the cells have been transformed with a polynucleotide vector of the present invention, the HO-1 gene is overexpressed in those cells. In a preferred embodiment, the expression vector is integrated into the genome of the target cell to provide stable overexpression of an HO-1 enzyme in those cells.

Polynucleotide expression vectors of the present invention can be introduced into cells or tissues by *in vivo* or *ex vivo* means. Preferably, the polynucleotide vectors are introduced *in vivo* by a variety of viral and non-viral means. Suitable nonviral means include, for example, polynucleotide complexed with cationic lipids, and encapsulation of the polynuceotides in liposome vesicles. Suitable viral vectors can be prepared from retroviruses, such as murine leukemia viruses and HIV-derived vectors , adeno-associated viruses, and adenovirus. Other suitable viral vectors are known in the art and are contemplated in the present invention.

Polynucleotide vectors of the present invention for HO-1 gene therapy can be administered to a person in need of such treatment according to standard procedures and methods known in the art. Preferably, the polynucleotide vectors are administered in a biologically compatible solution by direct injection or contact with the target cells and/or

tissue of the patient. For example, the subject vectors can be administered to protect kidney cells from damage from cisplatin and other agents, by injecting *in vivo* a polynucleotide expression vector directly in kidney tissue or by contacting kidney tissue *in vivo* with a polynucleotide vector or plasmid of the invention. In one embodiment, the polynucleotide vector can be delivered to the kidney via catheter inserted into the proximal tubule. The amount of vector to be administered can be readily determined by a person of ordinary skill in the art.

HO-1 polynucleotide sequences included within the scope of the present invention include those sequences that encode HO-1 polypeptide that are derived from animals, including mammals. Preferably, the sequence is a human sequence of HO-1.

HO-1 polynucleotide sequences contemplated in the subject invention also include HO-1 genes having the natural sequence, as well as allelic variants and degenerate variants that encode an enzyme having HO-1 activity. The subject invention also concerns polynucleotide vectors and plasmids comprising polynucleotide sequences that encode a fragment or variant of an active HO-1 protein as long as that fragment or variant has substantially the same biological activity as the natural full length protein. Such fragments and variants can be readily prepared using standard methods. Thus, contemplated in all aspects of the invention are HO-1 polypeptides, and the polynucleotide sequences that encode them, as well as biologically active fragments and variants. The vectors of the subject invention can also be designed for targeted delivery and/or expression of the HO-1 gene in kidney cells. Targeted expression can be achieved by using suitable polynucleotide regulatory control elements to control expression of vector linked genes in particular cells or types of cells.

The subject invention also concerns polynucleotide expression vectors and plasmids comprising a polynucleotide sequence that encodes a heme oxygenase-1 enzyme, or a biologically active fragment or variant thereof. An exemplified polynucleotide plasmid of the subject invention has been designated pcDNA3.1/HO-1 (Figure 1A). This plasmid was prepared by inserting an EcoRI/Xbal fragment that includes the HO-1 protein coding region into the pcDNA3.1/zeo plasmid. This vector can be prepared by using standard procedures well known in the art. It comprises a cytomegalovirus promoter/enhancer and the protein coding region of the HO-1 gene. The procedure disclosed in Abraham *et al.* (Abraham, Nader G., Jean-Louis da Silva,

Yan Lavrovsky, Robert A. Stolz, Attallah Kappas, Michael W. Dunn, Michael Laniado Schwartzman (1995) "Adenovirus-Mediated Heme Oxygenase-1 Gene Transfer Into Rabbit Ocular Tissues" *Investigative Ophthalmology & Visual Science* 36(11):2202-2210) can be used in constructing suitable vectors of the subject invention by substituting the well-known cytomegalovirus for the adenovirus described in the publication.

Preferably, the polynucleotide vectors contain regulatory elements that provide for high levels of expression of an HO-1 encoding polynucleotide in the cells. More preferably, the polynucleotide vectors include promoter and/or enhancer elements selected for high levels of expression of any operably linked genes, such as HO-1, in mammalian cells. In an exemplified embodiment, the polynucleotide vector comprises cytomegalovirus promoter and/or enhancer sequences operably linked to that region of the HO-1 gene that encodes an active form of the enzyme. Other promoter sequences, such as adeno-associated virus inverted terminal repeat sequences as described in published international application number PCT/US93/05310, are contemplated for use with the subject invention. Preferably, the polynucleotide vector also comprises a polynucleotide encoding selectable marker, such as Zeocin, neoR, thymidine kinase, beta-galactosidase, chloroamphenicol acetyl transferase (CAT), dihydrofolate reductase (DHFR) and the like, which allows one to select for those cells that are stably transformed by the polynucleotide vector. The vector can also comprise a reporter gene for detection of HO-1 expression.

The polynucleotide sequences, including polynucleotide vectors and plasmids, of the subject invention can be composed of either DNA or RNA. Nucleotide analogs that can replace the normal nucleotides found in DNA and RNA can also be used in the subject polynucleotides, vectors, and plasmids.

The subject invention also concerns cells and tissues transformed with a polynucleotide expression vector comprising a polynucleotide sequence which encodes a heme oxygenase-1 enzyme. Transformed cells in tissues contemplated within the scope of the invention include kidney, spinal cord, brain, liver, lung, intestine and skin. Kidney cells that can be transformed according to the subject invention include, for example, human proximal tubule cells.

The subject invention also concerns polynucleotides comprising a first polynucleotide sequence that encodes an HO-1 protein, or a biologically active fragment

or variant thereof, in combination with a second polynucleotide sequence that encodes a protein having anti-oxidant activity. Examples of anti-oxidant proteins that can be encoded by the second polynucleotide and that are contemplated within the scope of the invention include superoxide dismutase (SOD) polypeptides. In a preferred embodiment, the second polynucleotide sequence encodes a mutant manganese SOD protein that lacks or that has decreased product inhibition as compared to wild type SOD. In another preferred embodiment, the polynucleotide encoding the SOD is operatively linked to a polynucleotide leader sequence that targets the polynucleotide to mitochondria. Most preferably, the second polynucleotide sequence encodes a mutant manganese SOD protein that lacks or that has decreased product inhibition as compared to wild type SOD and is operatively linked to a polynucleotide leader sequence that targets the polynucleotide to mitochondria The first and second polynucleotides can be operatively linked on a single vector or they can be present on separate vectors. Preferably, the first and second polynucleotides are operatively linked on a single vector. The order of the first and second polynucleotides on the vector is unimportant as long as proper protein expression from each of the protein encoding polynucleotides can be achieved once the vector is delivered to a target cell. In a preferred embodiment, the first and second polynucleotides are operatively linked on a single vector wherein an internal ribosome entry site (IRES) (Rees, S., Coote, J., Stables, S., Harris, S. and Lee, M.G., (1996) "Bicistronic vector for the creation of stable mammalian cell lines that predisposes all antibiotic-resistant cells to express recombinant protein" *Biotechniques* 20:102-110; CLONTECH 1998/99 catalog, Palo Alto, CA) sequence is provided between them. The polynucleotide vectors of the invention comprising first and/or second polynucleotides can further comprise regulatory sequences that enhance or promote expression of the polynucleotide sequences encoding the polypeptides. Preferably, the regulatory sequences are sequences that can be induced to upregulate expression of the encoded proteins in the target cell. More preferably, the regulatory sequences upregulate expression of the encoded proteins in the target cell in response to a physiological phenomena, such as, for example, an inflammatory or immune response in the host animal. Suitable regulatory sequences include promoter sequences, enhancer sequences, and intron sequences.

The present invention also concerns methods for regulating or inhibiting cellular

apoptosis. In one embodiment, apoptosis is regulated or inhibited in a target cell by expression of a polynucleotide sequence that encodes an HO-1 polypeptide. In a preferred embodiment of the subject invention, a polynucleotide encoding an HO-1 polypeptide is delivered to and expressed in a target cell. In another embodiment, the expression of an endogenous polynucleotide encoding an HO-1 polypeptide in a target cell is induced by administering an agent capable of inducing endogenous HO-1 expression. As shown in Figure 7, pre-treatment of human proximal tubule cells with hemin to induce HO-1 expression protects cells against cisplatin mediated apoptosis as measured by the Annexin I assay.

The present invention also concerns methods for enhancing transplantation success of tissues and organs in xenografts and allografts. In one embodiment, transplantation success of a xenograft or allograft in a host animal is enhanced by expression of a polynucleotide sequence that encodes an HO-1 polypeptide. In a preferred embodiment of the subject invention, a polynucleotide encoding an HO-1 polypeptide is delivered to and expressed in a target cell of the transplanted tissue or organ. In another embodiment, the expression of an endogenous polynucleotide encoding an HO-1 polypeptide in a target cell of the transplanted tissue or organ is induced by administering an agent capable of inducing endogenous HO-1 expression. Tissue and organs contemplated for transplantation include, but are not limited to, heart, lung, liver, kidney, skin, spinal cord and fetal tissues.

The present invention also concerns methods for treating or preventing conditions such as atherosclerosis, acute respiratory distress conditions, ischemia-reperfusion injury, radiation-induced injury and other conditions that involve oxidative stress. The subject invention also concerns methods for treating cadmium exposure which causes nephrotoxicity. In one embodiment, a target cell is treated or induced to express a polynucleotide sequence that encodes an HO-1 polypeptide. In a preferred embodiment of the subject invention, a polynucleotide encoding an HO-1 polypeptide is delivered to and expressed in a target cell. In another embodiment, the expression of an endogenous polynucleotide encoding an HO-1 polypeptide in a target cell is induced by administering an agent capable of inducing endogenous HO-1 expression.

The subject invention also pertains to polynucleotides having sequences that are antisense to polynucleotides that encode HO-1, or a fragment or variant thereof. The

antisense polynucleotides include those that are antisense to both DNA encoding HO-1, as well as RNA transcribed from that DNA. The antisense sequences of the subject invention can be delivered to and expressed in target cells, such as cancer cells, for which one would like to decrease or prevent expression of HO-1. Thus, the subject invention also concerns methods for cancer therapy by inhibiting expression of HO-1 in targeted cancer cells using the antisense sequences of the invention.

All publications cited herein are hereby incorporated by reference.

Materials and Methods

Transfection:

Cells were transfected using the lipofectamine method (Life Technologies, Gaithersburg, MD).

LDH assay:

Transfected HEK 293 cells were plated in 24 well plates and incubated at 37°C for 24 hours. The cells were exposed to CP(100μM). Sixteen hours after exposure, 100μl of supernatant was transferred in 96 wll plates and 200 μl of working solution containing diaphorase, NAD^+, iodo-tetrazolium chloride and sodium lactate were added. The enzyme reaction was stopped by the addition of 50 μl of 1N HCl. The absorbance of samples at 492 nm and 620 nm was measured. The percent specific LDH release was calculated by the absorbance of treated samples over control and Triton-X treated samples.

Assessment of mitochondrial viability by the MTT assay:

Transfected cells were plated into 96 well plates and incubated for 3 days, followed by exposure to media containing PBS (control) or CP (100μM) for 16 hours. Ten microliters of 5 mg/ml MTT stock solution was added and cells were incubated for 4 hours. One hundred μl of acidic isopropanol (0.04N HCl in isopropanol) was added. The absorbance of samples at 550nm and 690nm was measured. Results are expressed as percentage change in absorbance over control cells in each group.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example - 1

In order to show that cells transfected with the polynucleotide expression vector of the subject invention provided for increased expression of the HO-1 gene, approximately 300 base pairs of nucleotides were removed from the 3' prime noncoding sequence of the HO-1 cDNA on the vector. Because the heterologous HO-1 mRNA will be shorter in size than an endogenous HO-1 mRNA, it was possible to distinguish expression of endogeneous HO-1 mRNA from heterologous HO-1 mRNA that is transcribed from the subject vector in the cells. Figure 1B shows a Northern blot, using the pcDNA3.1/Zeo plasmid as a probe, of RNA from both untreated human renal tubule cells (lane 1) and human renal tubule cells transformed with an expression vector having the shorter HO-1 cDNA according to the subject invention (lane 2). As can be seen in lane 1, the untreated cells express only endogenous HO-1 mRNA. Lane 2 of Figure 1B shows that the transformed cells express the endogenous HO-1 mRNA and overexpress the shorter HO-1 mRNA transcribed from the subject vector.

Example - 2

Human proximal tubule cells were transfected with either a vector lacking the HO-1 gene or with vector that included a polynucleotide sequence encoding the HO-1 enzyme. Cells were then exposed to varying concentrations of cisplatin (10 μM, 50 μM, and 100 μM). Lactate dehydrogenase (LDH) release was then measured from the cells. Release of lactate dehydrogenase from cells reflects the damage to the cells as a result of cytotoxicity of cisplatin. Thus, the greater percentage release of LDH from cells correlates with greater cytotoxicity associated with cisplatin treatment. Figure 2 shows that cells expressing the HO-1 gene exhibited an approximately 30% decrease in LDH release at both the 50 μM and 100 μM concentrations of cisplatin. Thus, overexpression of HO-1 in these cells provided a cytoprotective effect against damage from cisplatin as measured by LDH release from the cells.

Example - 3

Figure 3 shows the morphology of renal tubule cells either untreated (Fig. 3A) or treated with cisplatin (Fig. 3B) or pretreated with hemin (an inducer of HO-1 expression) followed by cisplatin exposure (Fig. 3C). As can be seen in the micrographs, cells exposed to cisplatin alone show a damaged morphology. In contrast, cells pretreated with hemin and then exposed to cisplatin retain morphology and cell structure strikingly similar to untreated control cells. Thus, this provides evidence that expression of HO-1 enzyme in these cells can confer a cytoprotective effect from damage due to treatment with cisplatin and other agents which cause heme related damage to cells.

Example - 4

As shown in Figure 6, the cytoprotective role of prior induction of HO-1 in cytotoxicity induced by cisplatin (CP) in human proximal tubule cells (HPTC) was evaluated. Confluent HPTC were pretreated for 2h with low dose hemin (5μM), a potent inducer of HO-1, 12h prior to exposure to CP (100μM) for 12-24h. Cytotoxicity was determined by the LDH assay and expressed as % LDH release over control. A 6- and 4-fold increase in HO-1 protein expression occurred at 12 and 24h, respectively, in response to hemin treatment. Prior induction of HO-1, by hemin pretreatment, significantly attenuated CP-induced cytotoxicity by 60% (CP alone: 16.1±2.7%; hemin+CP; 6.35±1.6%; $p<0.05$)(n=4) at 12h. The specificity of the cytoprotective effect of HO-1 was confirmed by co-treatment of cells with zinc protoporphyrin (ZnPP) (5μM), an inhibitor of HO-1. Inhibition of HO-1 with ZnPP resulted in reversal of the cytoprotective effect conferred by hemin pretreatment (hemin+CP+ZnPP: 15.4±1.8%). Hemin and ZnPP alone demonstrated no significant cytotoxicity. The protective effect of hemin pretreatment was also seen after 24h of exposure to CP (100μM) (CP:36.5%; Hemin+CP: 17.6%, $p<0.05$).

These data demonstrate a protective role of prior induction of HO-1 in CP-induced cytotoxicity in HPTC. Measures targeted to overexpress HO-1 may have implications in designing possible therapeutic approaches to attenuate CP nephrotoxicity while still taking advantage of the beneficial antineoplastic properties of CP as a treatment for cancer.

Example - 5

The beneficial effects of the anticancer drug, cisplatin, are mitigated by significant nephrotoxicity. Studies were performed to manipulate the HO-1 gene at the molecular level resulting in selective overexpression in our model of cisplatin-induced cytotoxicity. HEK293 cells were transfected with a vector containing the entire protein coding region of the human HO-1 gene (HHO) under the promoter/enhancer of cytomegalovirus (pcDNA3.1/HHO). The vector alone was used as a transfection control. Overexpression was confirmed by northern and immunoblot analyses which revealed a ~40 fold and ~10 fold increase in mRNA and protein, respectively, over control cells (Figure 1B and 1D). Cells were exposed to cisplatin (100μM) for 16 hours and cytotoxicity assessed by phase contrast microscopy, LDH assay and the MTT assay. Morphological indices of injury such as cell rounding, vacuolization, and detachment were significantly reduced in cells with elevated levels of HO-1 compared to cells transfected with the vector alone. These changes were accompanied by a significant attenuation of cisplatin-induced cytotoxicity by 68% in such cells as determined by LDH release (Vector alone: 20+/-6.8%, HO-1 overexpression: 6.6+/-2.82, $p<0.05$, n=4) (see Figure 5). Protection from cell injury was also confirmed by the MTT assay. This assay requires the presence of active mitochondrial dehydrogenases to reduce the dye MTT to formazan, a purple colored dye. Exposure to cisplatin (100μM) for 16 hours resulted in significant impairment in the ability of cells transfected with vector alone to reduce MTT compared to HO-1 overexpressing cells (Vector alone, 16.6 +/-2.8 vs. HO-1 overexpression, 5.26+/-2.3%; $p<0.05$, n=3) (Figure 8). Results are expressed as percentage change in MTT over control untreated cells in each group. Treatment of cells with a competitive inhibitor, zinc protoporphyrin, reversed the cytoprotective effects of HO-1. These data demonstrate the protective role of HO-1 in cisplatin nephrotoxicity and in high risk settings of acute renal failure.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

1. A polynucleotide expression vector comprising a polynucleotide sequence encoding a heme oxygenase -1 (HO-1) enzyme, or a biologically active fragment or variant thereof.

2. The polynucleotide expression vector according to claim 1, wherein said vector comprises the amino acid coding region of the polynucleotide sequence shown in Figure 4B.

3. The polynucleotide expression vector according to claim 1, wherein said vector comprises regulatory sequences operably linked with said polynucleotide sequence encoding said heme oxygenase-1 enzyme, wherein said regulatory sequences promote expression of said polynucleotide sequence.

4. The polynucleotide expression vector according to claim 3, wherein said regulatory sequence comprises a promoter or enhancer sequence.

5. The polynucleotide expression vector according to claim 4, wherein said promoter or enhancer sequence is derived from cytomegalovirus.

6. The polynucleotide expression vector according to claim 1, wherein said vector is derived from a viral vector.

7. The polynucleotide expression vector according to claim 6, wherein said viral vector is selected from the group consisting of adeno-associated virus, retrovirus, and adenovirus.

8. The polynucleotide expression vector according to claim 1, wherein said vector comprises a polynucleotide sequence encoding a heme oxygenase-1 enzyme comprising the amino acid sequence shown in Figure 4A.

9. A method for protecting cells or tissue from damage, said method comprising transforming said cells or tissue with the polynucleotide expression vector of claim 1 to express a heme oxygenase-1 enzyme in said cells or tissue.

10. A cell transformed with the polynucleotide expression vector of claim 1.

11. The cell of claim 10, wherein said cell is a kidney cell.

12. The cell of claim 11, wherein said kidney cell is a human renal proximal tubal cell.

13. A tissue transformed with the polynucleotide expression vector of claim 1.

14. The tissue of claim 13, wherein said tissue is from kidney.

15. A method for treating a patient, wherein said patient is to receive or is receiving treatments with agents that cause or are associated with heme protein-related damage or oxidative-related damage to cells or tissues in said patient, said method comprising transforming cells or tissue with a polynucleotide, wherein expression of said polynucleotide provides a heme oxygenase-1 enzyme in said cells or tissue.

16. The method according to claim 15, wherein said agent is an agent for treating a condition selected from the group consisting of infection, cancer, and immunosuppression.

17. The method according to claim 15, wherein said agent is a radiocontrast agent.

18. The method according to claim 15, wherein said cell is selected from the group consisting of kidney cell, brain cell, liver cell, lung cell, skin cell, gastrointestinal cell, and spinal cord cell.

19. The method according to claim 15, wherein said heme oxygenase-1 enzyme is overexpressed in said cell or tissue relative to a cell or tissue that has not been transformed with said polynucleotide.

20. The method according to claim 15, wherein said polynucleotide is provided in the form of an expression vector.

21. The method according to claim 20, wherein said expression vector comprises a suitable promoter sequence to promote expression of said heme oxygenase-1 enzyme in said cell or tissue.

22. The method according to claim 21, wherein said promoter is selected from the group consisting of cytomegalovirus promoters, adenovirus promoters and adeno-associated virus promoters.

23. The method according to claim 20, wherein said expression vector comprises an enhancer sequence.

24. The method according to claim 20, wherein said expression vector is selected from the group consisting of retrovirus vectors, adeno-associated virus vectors and adenovirus vectors.

25. The method according to claim 15, wherein said cell or tissue is transformed *in vivo* with said polynucleotide.

26. The method according to claim 15, wherein said cell or tissue is transformed *ex vivo* with said polynucleotide.

27. The method according to claim 20, wherein said expression vector is pcDNA 3.1/Zeo.

28. A polynucleotide molecule, wherein said polynucleotide comprises a first polynucleotide sequence encoding a heme oxygenase-1 protein, or a biologically active fragment or variant thereof, and a second polynucleotide sequence encoding a protein having antioxidant activity.

29. The polynucleotide according to claim 28, wherein said second polynucleotide sequence encodes a superoxide dismutase protein.

30. The polynucleotide according to claim 29, wherein said superoxide dismutase protein encoded by said second polynucleotide is a mutant superoxide dismutase protein that exhibits decreased product inhibition as compared to wild-type superoxide dismutase protein.

31. The polynucleotide according to claim 28, wherein said second polynucleotide sequence encoding said superoxide dismutase protein is operatively linked to a polynucleotide leader sequence that targets said polynucleotide to mitochondria.

32. The polynucleotide according to claim 28, wherein an internal ribosome entry site sequence is provided between said first and second polynucleotide sequences.

33. The polynucleotide according to claim 28, wherein said polynucleotide comprises a regulatory sequence that enhances or promotes expression of said first or second polynucleotide sequences.

34. The polynucleotide according to claim 33, wherein said regulatory sequence upregulates expression of said first or second polynucleotide sequences in response to a physiological response.

35. The polynucleotide according to claim 34, wherein said physiological response is selected from the group consisting of an inflammatory response and an immune response.

36. The polynucleotide according to claim 33, wherein said regulatory sequences are selected from the group consisting of promoter sequences, enhancer sequences and intron sequences.

37. A polynucleotide composition, wherein said polynucleotide composition comprises a first polynucleotide molecule encoding a heme oxygenase-1 protein, or a biologically active fragment or variant thereof; and a second polynucleotide molecule comprising a polynucleotide sequence encoding a protein having antioxidant activity.

38. A method for regulating or inhibiting cellular apoptosis, said method comprising transforming a cell with the polynucleotide expression vector of claim 1 to express a heme oxygenase-1 enzyme in said cell.

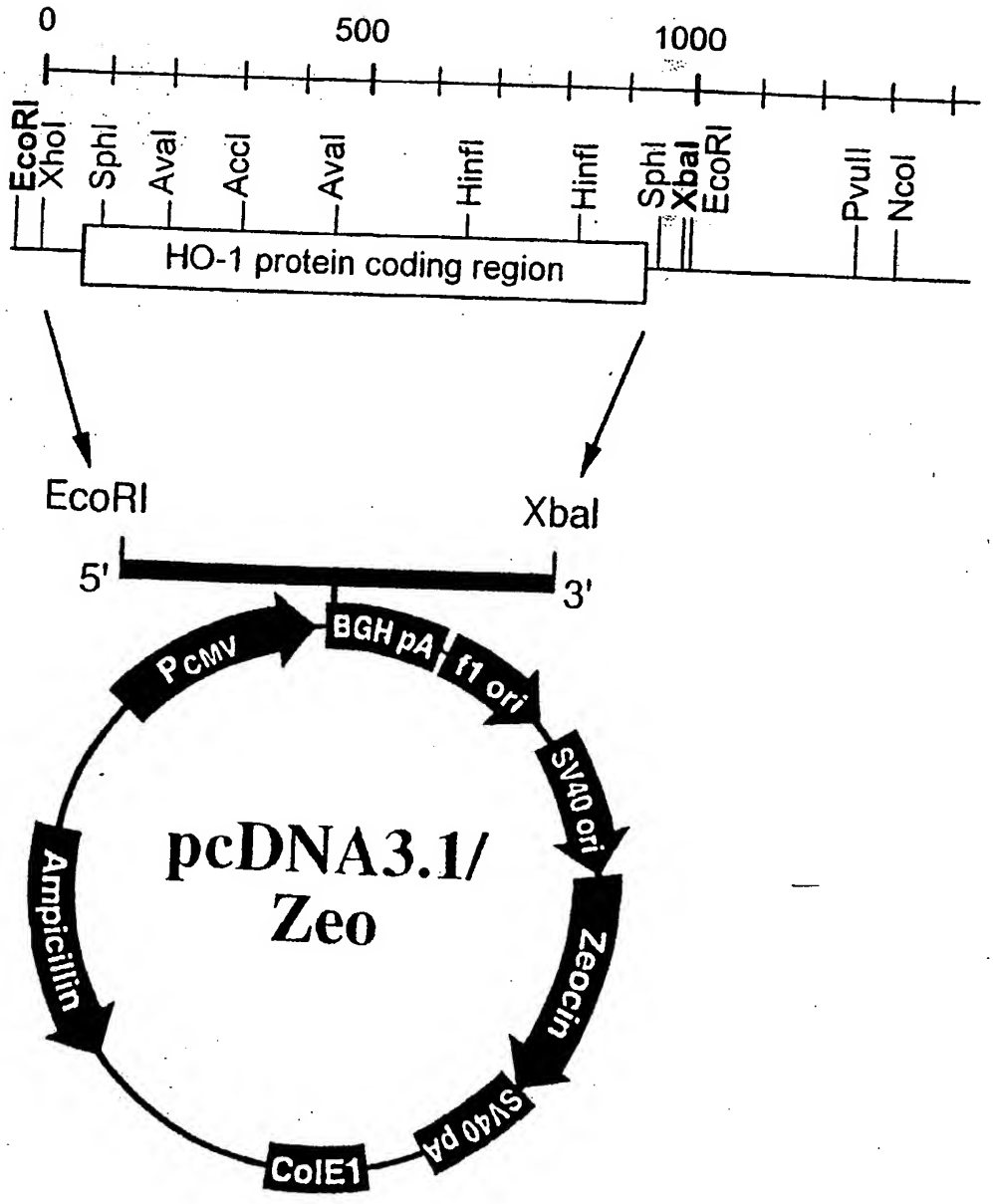

0
500
1000
EcoRI
XhoI
SphI
AvaI
AccI
AvaI
HinfI
HinfI
SphI
XbaI
EcoRI
PvuII
NcoI
HO-1 protein coding region
EcoRI
XbaI
5'
3'
PCMV
BGH pA
f1 ori
SV40 ori
Zeocin
SV40 pA
ColE1
Ampicillin
pcDNA3.1/
Zeo

FIG. 1A

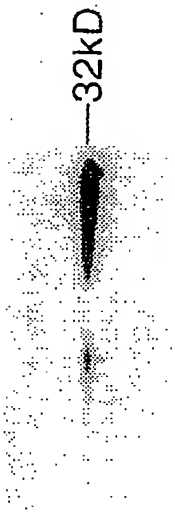


FIG. 1D

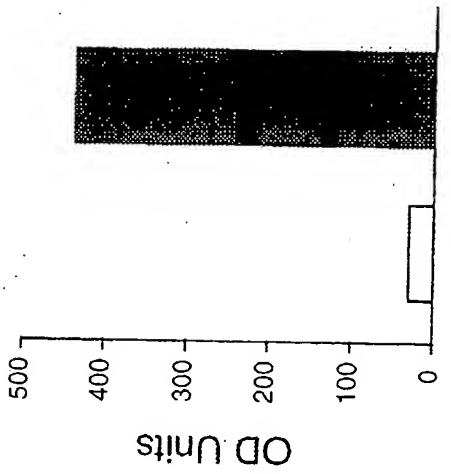


FIG. 1E

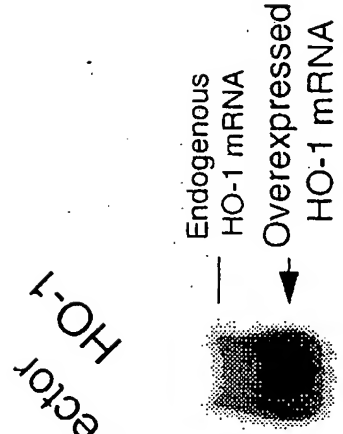


FIG. 1B

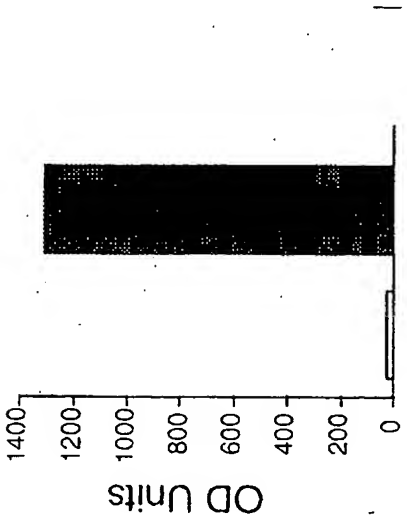


FIG. 1C

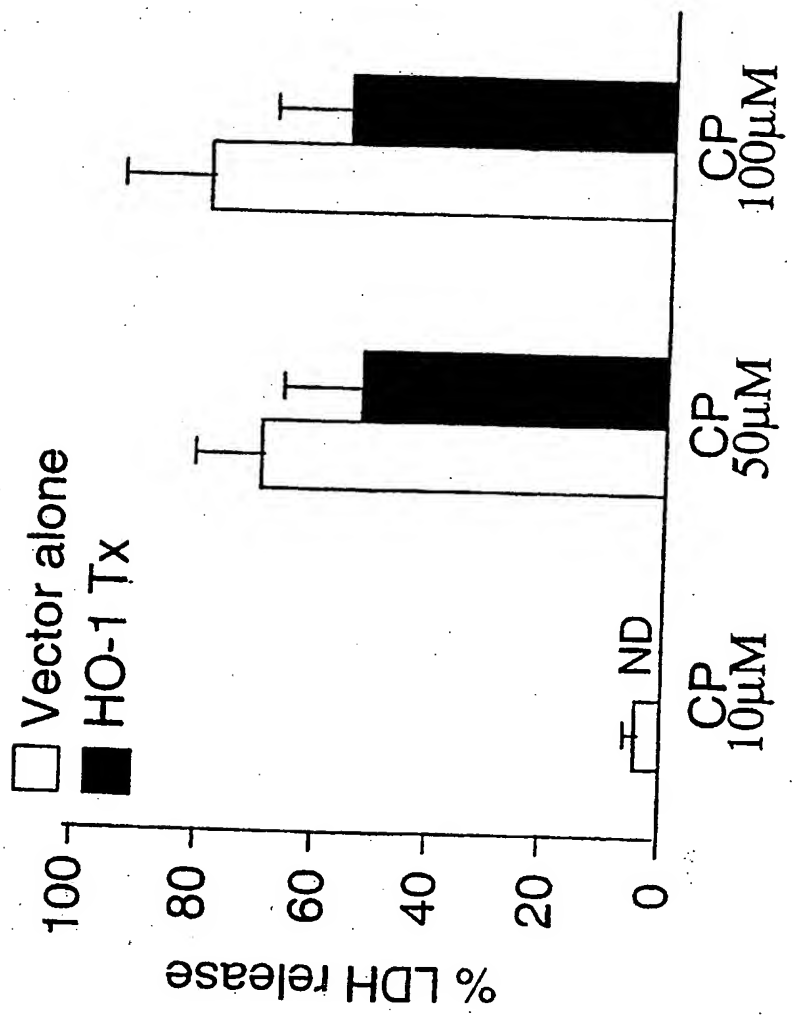
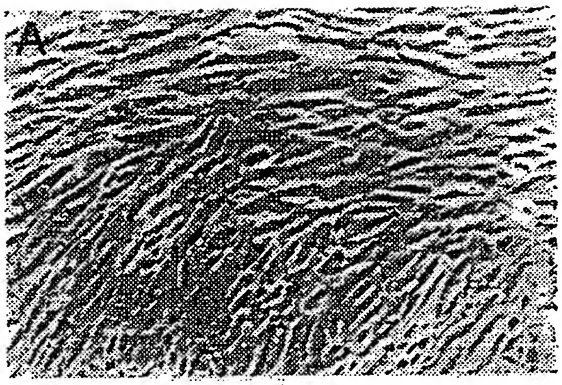

% LDH release
100
80
60
40
20
0
Vector alone
HO-1 Tx
ND
CP 10μM
CP 50μM
CP 100μM

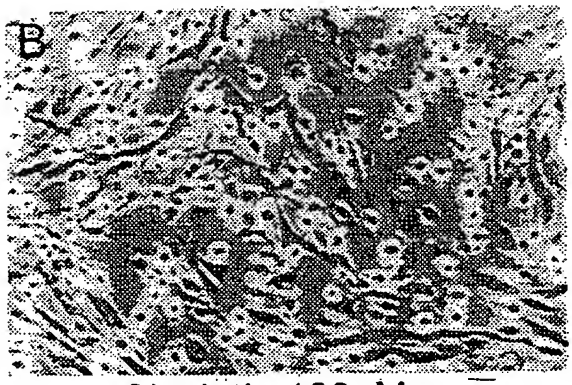
FIG. 2

FIG. 3A



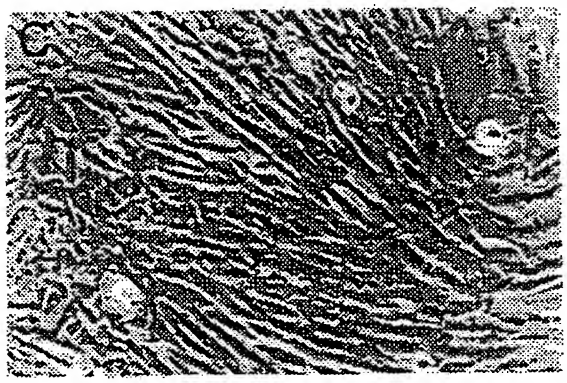
Control

FIG. 3B



Cisplatin 100μM

FIG. 3C



Hemin pretreated +Cisplatin 100μM

translation="MERPQPDSMPQDLSEALKEATKEVHTQAENAEFM
RNFQKGQVTRDGFKLVMASLYHIYVALEEEIERNKESPVFAPVY
FPEELHRKAALEQDLAFWYGPRWQEVIPYTPAMQRYVKRLHE
VGRTEPELLVAHAYTRYLGDLSGGQVLKKIAQKALDLPSSGEG
LAFFTFPNIASATKFKQLYRSRMNSLEMTPAVRQRVIEEAKTAF
LLNIQLFEELQELLTHDTKDQSPSRAPGLRQRASNKVQDSAPV
ETPRGKPPLNTRSQAPLLRWVLTLSFLVATVAVGLYAM"

FIG. 4A

1 tcaacgcctg cctcccctcg agcgtcctca gcgcagccgc cgcccgcgga gccagcacga
61 acgagcccag caccggccgg atggagcgtc cgcaacccga cagcatgccc caggatttgt
121 cagaggccct gaaggaggcc accaaggagg tgcacaccca ggcagagaat gctgagttca
181 tgaggaactt tcagaagggc caggtgaccc gagacggctt caagctggtg atggcctccc
241 tgtaccacat ctatgtggcc ctggaggagg agattgagcg caacaaggag agcccagtct
301 tcgcccctgt ctacttccca gaagagctgc accgcaaggc tgccctggag caggacctgg
361 ccttctggta cgggcccccgc tggcaggagg tcatccccta cacaccagcc atgcagcgct
421 atgtgaagcg gctccacgag gtgggggcgca cagagcccga gctgctggtg gcccacgcct
481 acacccgcta cctgggtgac ctgtctgggg gccaggtgct caaaaagatt gcccagaaag
541 ccctggacct gcccagctct ggcgagggcc tggccttctt caccttcccc aacattgcca
601 gtgccaccaa gttcaagcag ctctaccgct cccgcatgaa ctccctggag atgactcccg
661 cagtcaggca gagggtgata gaagaggcca agactgcgtt cctgctcaac atccagctct
721 ttgaggagtt gcaggagctg ctgacccatg acaccaagga ccagagcccc tcacgggcac
781 cagggcttcg ccagcgggcc agcaacaaag tgcaagattc tgccccccgtg gagactccca
841 gagggaagcc cccactcaac acccgctccc aggctccgct tctccgatgg gtccttacac
901 tcagctttct ggtggcgaca gttgctgtag ggctttatgc catgtgaatg caggcatgct
961 ggctcccagg gccatgaact ttgtccggtg gaaggccttc tttctagaga gggaattctc
1021 ttggctggct tccttaccgt gggcactgaa ggctttcagg gcctccagcc ctctcactgt
1081 gtccctctct ctggaaagga ggaaggagcc tatggcatct tccccaacga aaagcacatc
1141 caggcaatgg cctaaacttc agagggggcg aaggggtcag ccctgccctt cagcatcctc
1201 agttcctgca gcagagcctg gaagacaccc taatgtggca gctgtctcaa acctccaaaa
1261 gccctgagtt tcaagtatcc ttgttgacac ggccatgacc actttccccg tgggccatgg
1321 caatttttac acaaacctga aaagatgttg tgtcttgtgt ttttgtctta tttttgttgg
1381 agccactctg ttcctggctc agcctcaaat gcagtatttt tgttgtgttc tgttgttttt
1441 atagcagggt tggggtggtt tttgagccat gcgtgggtgg ggagggaggt gtttaacggc
1501 actgtggcct tggtctaact tttgtgtgaa ataataaaca acattgtctg

FIG. 4B

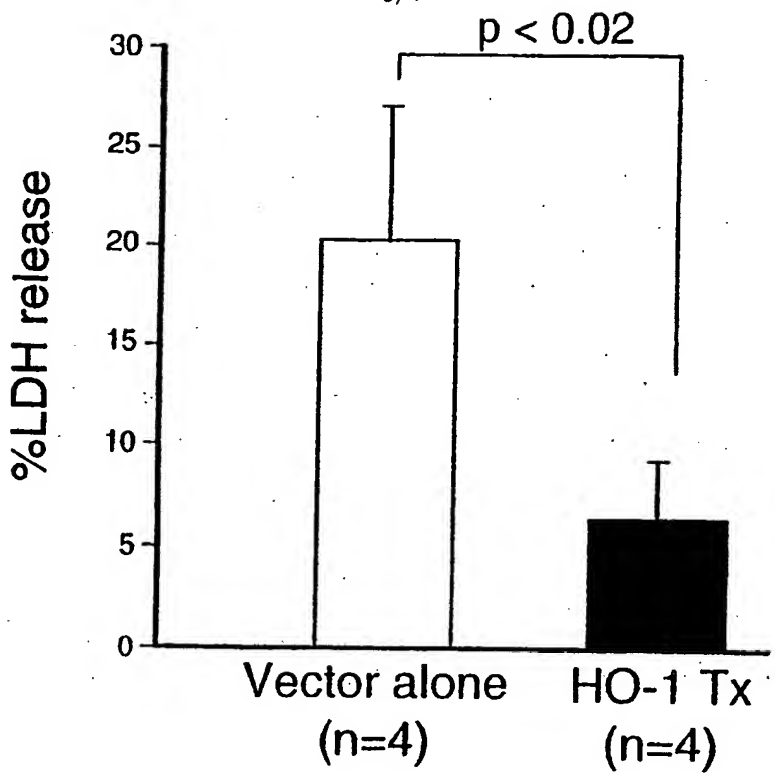

p < 0.02
%LDH release
30
25
20
15
10
5
0
Vector alone
(n=4)
HO-1 Tx
(n=4)

FIG. 5

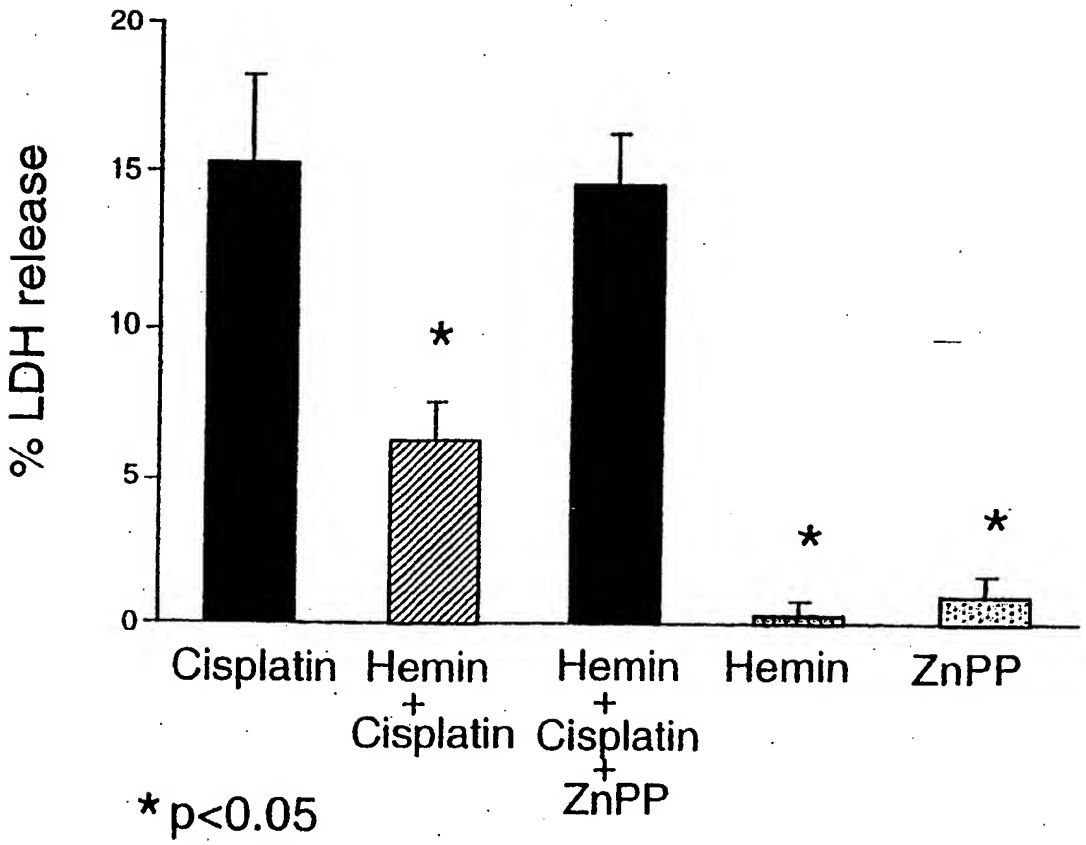

% LDH release
20
15
10
5
0
*
*
*
Cisplatin
Hemin + Cisplatin
Hemin + Cisplatin + ZnPP
Hemin
ZnPP
* p<0.05

FIG. 6

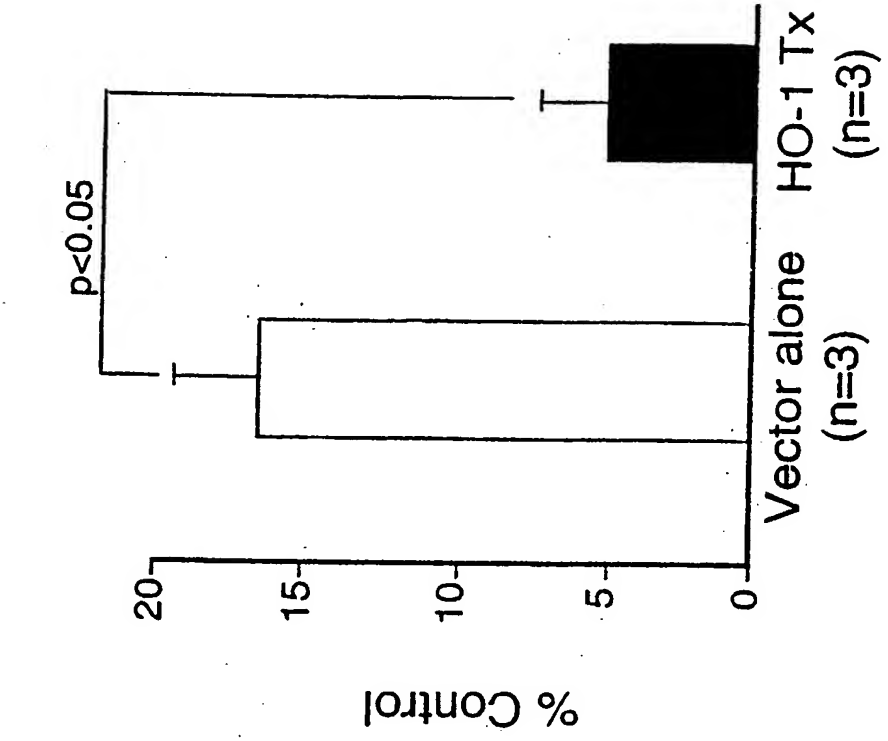

% Control
20
15
10
5
0
p<0.05
Vector alone (n=3)
HO-1 Tx (n=3)

FIG. 8

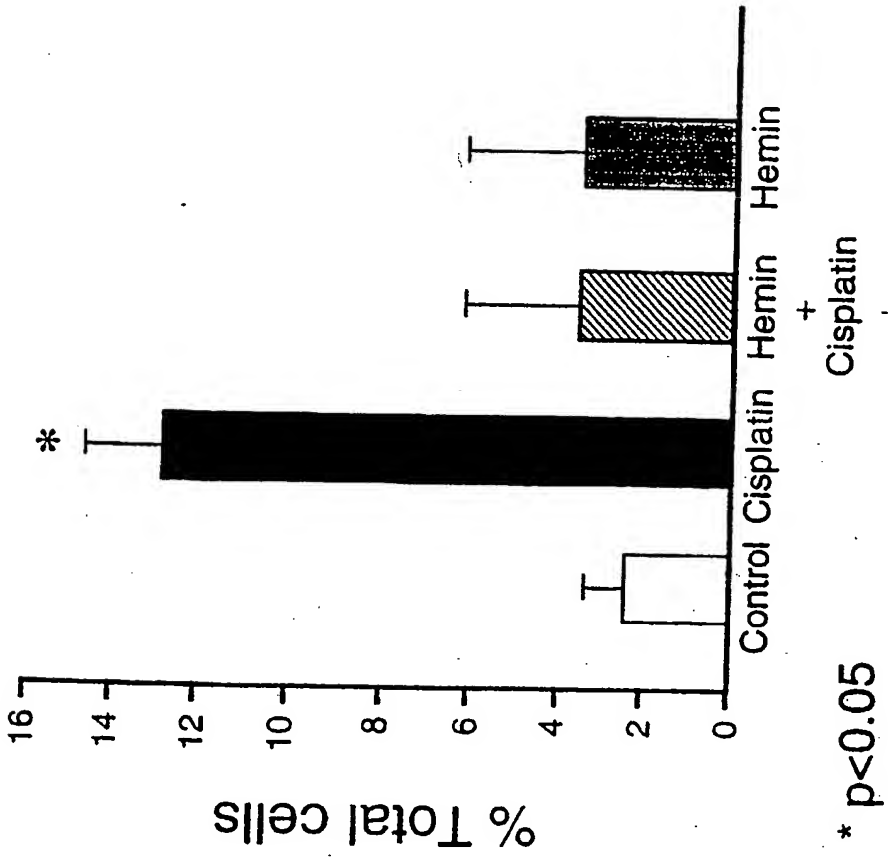

% Total cells
16
14
12
10
8
6
4
2
0
*
Control
Cisplatin
Hemin
Hemin + Cisplatin
* p<0.05

FIG. 7

INTERNATIONAL SEARCH REPORT

Interna l Application No
PCT/US 98/23270

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 C12N15/86 C12N15/62 C12N5/10 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABRAHAM, N.G. ET AL.: "Adenovirus-mediated heme oxygenase-1 gene transfer into rabbit ocular tissues" INVESTIGATIVE OPHTHALMOLOGY & VISUAL SCIENCE, vol. 36, no. 11, October 1995, pages 2202-2210, XP002086227 cited in the application	1-10,13
Y	see the whole document	11,12, 14-26, 28,29, 32-37

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 April 1999	07. 05. 99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Donath, C

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INTERNATIONAL SEARCH REPORT

Intern. al Application No
PCT/US 98/23270

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ABRAHAM, N.G. ET AL.: "Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: protective effect against heme and hemoglobin toxicity" PROC.NATL.ACAD.SCI.USA, vol. 92, no. 15, 18 July 1995, pages 6798-6802, XP002100374	1-5,8, 10,13
Y	see page 6798 - page 6801 'Materials and Methods' and 'Results' ---	15-23, 25,26, 28,29, 32-37
X	LEE, P.J. ET AL.: "Overexpression of heme oxygenase-1 in human pulmonary epithelial cells results in cell growth arrest and increased resistance to hyperoxia" PROC.NATL.ACAD.SCI.USA, vol. 93, September 1996, pages 10393-10398, XP002100375 cited in the application see page 10393 - page 10396 'Materials and Methods' and 'Results' ---	1,3,4,9, 10
Y	AGARWAL, A. ET AL.: "Induction of heme oxygenase in toxic renal injury: A protective role in cisplatin nephrotoxicity in the rat " KIDNEY INTERNATIONAL, vol. 48, no. 4, October 1995, pages 1298-1307, XP002100376 cited in the application see the whole document ---	11,12, 14-26
X	DENNERY, P.A. ET AL.: "Heme oxygenase-mediated resistance to oxygen toxicity in hamster fibroblasts" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 23, 6 June 1997, pages 14937-14942, XP002100598	1-5,8, 10,13
Y	see page 14939 - page 14942 'Results and Discussion' ---	15-23, 25,26, 28,29, 32-37
Y	WO 92 07935 A (THE SCRIPPS RESEARCH INSTITUTE) 14 May 1992 see page 6, line 26 - page 7, line 22 see page 23, line 21 - page 26, line 11 see page 55, line 5 - page 59, line 4 see page 66, line 13 - page 70, line 1 --- -/--	28,29, 32-37

1

INTERNATIONAL SEARCH REPORT

Interr. al Application No
PCT/US 98/23270

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YOSHIDA, T. ET AL.: "Human heme oxygenase cDNA and induction of its mRNA by hemin" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 171, no. 3, 1 February 1988, pages 457-461, XP002100377 cited in the application see figure 4 -----	2,8

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte .tional application No.
PCT/US 98/23270

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 15-25, 27 and 38 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern: al Application No
PCT/US 98/23270

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9207935 A	14-05-1992	AU 8947791 A	26-05-1992